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Second generation analysis of antinuclear antibody (ANA) by combination of screening and confirmatory testing

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Abstract

Background: For the serological diagnosis of systemic autoimmune rheumatic diseases, a two-tier approach starting with sensitive antinuclear antibody (ANA) detection by indirect immunofluorescence (IIF) on HEp-2 cells followed by characterization of positive findings with different immunoassays is recommended. To overcome drawbacks of this approach, we developed a novel technique allowing the combination of screening and simultaneous confirmatory testing. For the first time, this creates the basis for second generation ANA testing.

Methods: ANA and autoantibodies (autoAb) to double-stranded DNA (dsDNA), CENP-B, SS-A/Ro52, SS-A/Ro60, SS-B/La, RNP-Sm, Sm, and Scl-70 were determined by IIF and enzyme-linked immunosorbent assay (ELISA), respectively, and compared to simultaneous analysis thereof by second generation ANA analysis in patients with systemic lupus erythematosus (n=174), systemic

sclerosis (n=103), Sjögren's syndrome (n=46), rheumatoid arthritis (n=36), mixed and undetermined connective tissue diseases (n=13), myositis (n=21), infectious disease (n=21), autoimmune liver disease (n=93), inflammatory bowel disease (n=78), paraproteinemia (n=11), and blood donors (n=101).

Results: There was very good agreement of second generation ANA testing with classical one by IIF and ELISA regarding testing for ANA and autoAb to dsDNA, CENP-B, SS-B, RNP-Sm, Scl-70, SS-A/Ro52, and SS-A/Ro60 (Cohen's $\kappa > 0.8$). The agreement for anti-Sm autoAb was good ($\kappa = 0.77$). The differences of both approaches were not significant for autoAb to SS-B/La, RNP-Sm, Scl-70, SS-A/Ro60, and SS-A/Ro52 (McNemar's test, $p > 0.05$, respectively).

Conclusions: Second generation ANA testing can replace the two-tier analysis by combining IIF screening with multiplex confirmative testing. This addresses shortcomings of classical ANA analysis like false-negative ANA findings and lack of laboratory efficiency and standardization.

Keywords: antinuclear antibody; digital fluorescence; second generation ANA testing; standardization; systemic autoimmune rheumatic disease.

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Introduction

Antinuclear antibody (ANA) detection is one of the most used tests in routine autoimmune diagnostics within the two-tier serological diagnosis of systemic autoimmune rheumatic diseases (SARD) [1–4]. Further, ANA belongs to the classification criteria of systemic lupus erythematosus (SLE) and autoimmune hepatitis (AIH) [5, 6]. Indirect immunofluorescence (IIF) is still the recommended method for highly sensitive analysis of antibodies to cellular autoantigenic targets referred to as ANA [1, 7, 8]. Classical ANA screening by IIF on human epithelial type 2 cells (HEp-2 cells) offers more than 30 clinically relevant nuclear and cytoplasmic antigenic targets for autoantibody (autoAb) detection which is unsurpassed by any other ANA analysis so far. Several autoantigenic targets resulting in positive ANA findings are still to be elucidated and their discovery can aid in the serology of SARD [9]. Furthermore, ANA can be associated with disease activity and predate the onset of SARD [10–12].

However, autoAb screening by IIF has proven to be difficult to standardize and automate until recently [12–14]. As a matter of fact, the required confirmatory testing of positive IIF results by specific autoAb immunoassays, such as enzyme-linked immunosorbent assay (ELISA) or other solid-phase immunoassay creates further constraints in routine laboratories strained already by rising sample numbers and growing lack of experts in IIF interpretation nowadays [15, 16]. Another drawback of the two-tier ANA analysis is the possibility of false-negative ANA in particular for autoAb to Sjögren syndrome antigen A (SS-A/Ro) [2, 17]. However, despite enormous efforts to introduce one-step ANA testing by automated multiplex non-HEp-2 cell-based detection of autoAb, the aforementioned two-tier approach has been confirmed by expert consensus recently [1].

To overcome drawbacks of the recommended two-tier ANA testing, we developed a unique IIF reaction environment which combines classical ANA analysis on HEp-2 cells and multiplex detection of autoAb by microbead immunoassay simultaneously. Indeed, the possibility of quantitative ANA screening and multiplex confirmatory testing has been reported in independent assays, respectively. Our group has pioneered the use of digital fluorescence microscopy in autoAb testing by developing an automated interpretation technique providing standardization of autoAb interpretation by IIF including ANA pattern reading [18–20]. Furthermore, we set up a multiplex IIF assay for the simultaneous detection of autoAb employing fluorescent microbeads with chemically activated surfaces for autoantigen immobilization

[21]. Merging of both techniques provides the unique opportunity to perform ANA screening and multiplex confirmatory testing within one test. This creates the basis for second generation ANA analysis. The simple assay setting allows manual qualitative ANA assessment employing classical fluorescence microscopy. Using digital fluorescence, quantitative ANA screening and confirmatory testing can be run automatically as well.

This study is the first report of the clinical assessment of combined ANA screening and confirmatory testing by comparing second generation ANA analysis to routine immunoassays of the classical two-tier approach.

Materials and methods

Clinical sera and healthy controls

The study included 697 serum samples that were provided by the “Forum Interdisciplinare per la Ricerca sulle Malattie Autoimmuni” (FIRMA group), Department of Rheumatology, School of Health Sciences, University of Thessaly, and the Institute of Immunology, Technical University of Dresden, Germany (Table 1). Reference sera for specific autoAb were purchased from The Binding Site (Birmingham, UK) and In.vent Diagnostica (Hennigsdorf, Germany).

The diagnoses of the different SARDs have been performed according to their own specific classification criteria [5, 22–24].

The study was approved by the Local Ethics Committees (Comitato Etico Milano Area B del 08.07.2014, CS – GA-115565; Ethical Committee of the Technical University of Dresden, EK56022014) and complies with the World Medical Association Declaration of Helsinki regarding ethical conduct of research involving human subjects and/or animals. Aliquots of the sera stored at -20°C were used to detect antibody reactivity.

Detection of autoAb with antigen-specific ELISA

Autoantibodies to Sjögren syndrome antigen B (SS-B/La), centromere protein B (CENP-B), Smith proteins (Sm), ribonucleoproteins and Sm (RNP-Sm), double-stranded DNA (dsDNA), topoisomerase-1 (scleroderma 70 kDa extractable fragment, Scl-70), Sjögren syndrome 60 kDa antigen A (SS-A/Ro60), and Sjögren syndrome 52 kDa antigen A (SS-A/Ro52) were detected using commercially available antigen-specific ELISAs (Orgentec, Wiesbaden, Germany, GA Generic Assays, Dahlewitz, Germany) according to instructions of the manufacturers.

Determination of ANA by indirect immunofluorescence

ANA detection by IIF was performed by a commercially available assay according to the instructions of the manufacturer (GA Generic Assays) [25]. Briefly, patient and control sera were diluted 1/80 and incubated for 30 min at room temperature. After washing, a secondary antibody

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Table 1: Characteristics of patients and blood donors.

Diagnosis	n	Gender f/m	Median age (IQR)
Systemic autoimmune rheumatic diseases	393	329/64	49 (38–63)
Systemic lupus erythematosus	174	150/24	40 (31–46)
Systemic sclerosis	103	85/18	61 (51–70)
Sjögren’s syndrome	46	44/2	49 (38–57)
Rheumatoid arthritis	36	29/7	61 (46–67)
Undifferentiated/mixed connective tissue disease	13	12/1	40 (34–58)
Myositis	21	9/12	51 (36–59)
Infectious diseases	21	13/8	46 (39–59)
HCV infection	10	7/3	57 (45–64)
HBV infection	3	1/2	55 (70–35)
EBV infection	4	3/1	42 (35–44)
CMV infection	2	2/0	34 (27–40)
HIV infection	2	0/2	58 (46–69)
Paraproteinemia	11	4/7	80 (84–78)
Autoimmune liver disease	93	42/51	39 (22–55)
Primary biliary cirrhosis	3	3/0	67 (60–71)
Autoimmune hepatitis type 1	10	8/2	13 (12–15)
Autoimmune hepatitis type 2	10	10/0	11 (8–14)
Primary sclerosing cholangitis	70	21/49	45 (35–57)
Inflammatory bowel disease	78	46/32	48 (37–57)
Ulcerative colitis	58	31/27	49 (38–57)
Crohn’s disease	20	15/5	40 (32–55)
Blood donors	101	53/48	41 (33–52)

[fluorescein isothiocyanate (FITC) anti-human IgG] was added for 30 min. After another appropriate washing, wells were covered and then interpreted on a standard fluorescence microscope.

Multiplex detection of ANA by CytoBead ANA

CytoBead ANA (GA Generic Assays) is a multiplex IIF test that combines the screening of ANA on HEp-2 cells and their confirmation with multiplex microparticle immunoassay using 9 µm and 15 µm red fluorescent microbeads (excitation 610 nm/emission 690 nm). Fluorescent microbeads were covalently coated with SS-B, CENP-B, Sm, RNP-Sm, dsDNA, Scl-70, SS-A/Ro60, and SS-A/Ro52, respectively, as described elsewhere [21]. Further, glass slides with multi-compartment wells were employed to immobilize HEp-2 cells in the central compartment (Figure 1). For simultaneous confirmative testing, antigen-coated microparticles were immobilized in the four compartments around the central part of the well. In addition, a reference microbead population of 12 µm size emitting green fluorescence and not coated with antigen was immobilized in each peripheral compartment to aid in the discrimination of the antigen-coated microbead populations.

Patient and control sera were diluted 1/80 and incubated for 30 min at room temperature. After washing, secondary antibodies (FITC and Cy5-labelled anti-human IgG) were added in the presence of 4',6-diamidino-2-phenylindole (DAPI) for 30 min. After another appropriate washing, wells were covered for further evaluation.

For classical ANA screening, fluorescence patterns on HEp-2 cells were interpreted by standard fluorescence microscopy.

Automatic ANA reading was performed by the fluorescence interpretation systems AKLIDES™ (Medipan, Berlin/Dahlewitz, Germany) [25, 26]. Simultaneous binding of autoAb to their corresponding antigenic targets on the microparticles results in the appearance of a green fluorescent halo. For semi-quantitative analysis, the intensities of the fluorescence halos can be readily detected by standard fluorescence microscopy. Instead, the signal intensity of the halo can be quantified and simultaneously located to the appropriate microbead population by digital fluorescence reading [21]. This provides the basis for automatic simultaneous interpretation of ANA on HEp-2 cells and their confirmation on antigen-coated microbeads. As a matter of fact, the fluorescence intensity is proportional to the autoAb titre employing automatic reading [19].

Fully automated ANA screening and confirmatory analysis

Fully automated ANA screening and confirmatory testing was performed on the interpretation system Aklides™ employing novel mathematical software algorithms for pattern recognition, reported elsewhere [14, 18, 25]. Briefly, the systems is composed of a motorized inverse microscope (IX83, Olympus, Tokyo, Japan) with a motorized scanning stage (IM120, Märzhäuser, Wetzlar, Germany), 400 nm, 490 nm, and 635 nm light-emitting diodes (PrecisExcite, CoolLED, Andover, UK), and a charge-coupled device gray-scale camera (Zelos285, Kappa, Gleichen, Germany). The Aklides™ software that integrates modules for device and autofocus control, image analysis, and pattern recognition algorithms interprets images of stained

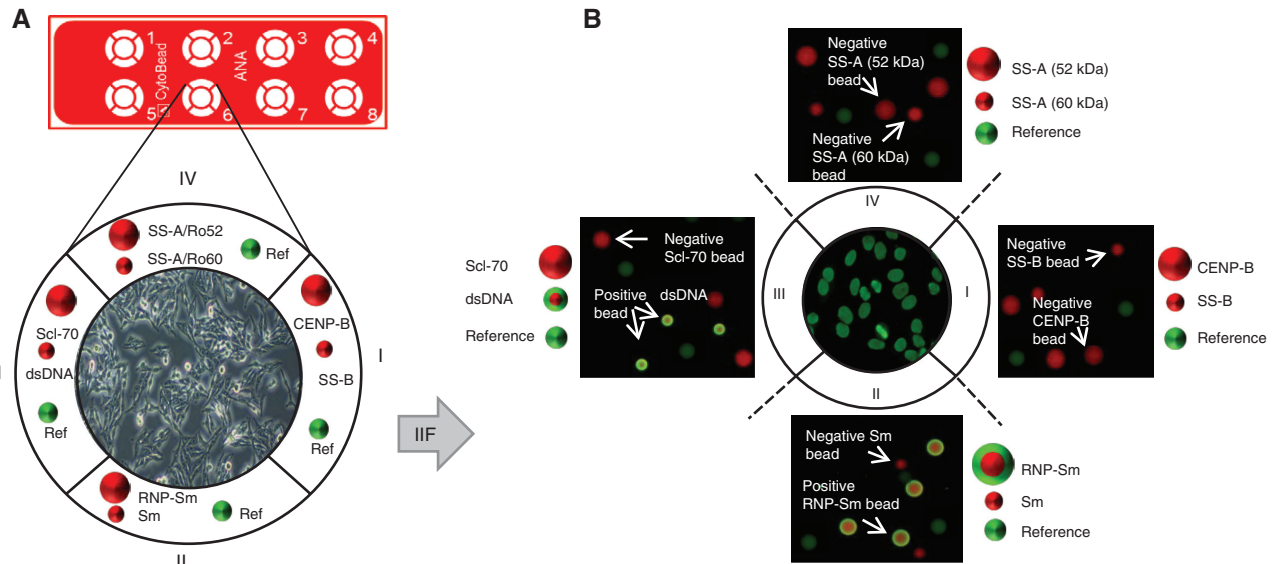


Figure 1: Assay principle of CytoBead ANA.

(A) Microscopic glass slides are employed as solid phase with fixed HEP-2 cells in the central part of wells for antinuclear antibody (ANA) screening and autoantigen-coated microparticles immobilized in the four peripheral compartments for confirmative testing by indirect immunofluorescence (IIF). (B) Example of an ANA-positive serum that shows a homogeneous fluorescence pattern on HEP-2 cells and a positive green fluorescence signal on RNP-Sm-coated microbeads (large red beads, compartment II) and dsDNA microbeads (small red beads, compartment III). Thus, this sample reveals ANA, anti-RNP-Sm, and anti-dsDNA autoantibody positivity.

HEP-2 cells at 400-fold (Olympus Planfluor, Olympus) and microbeads at 100-fold magnification. Single images were serially captured and stored in lossless compressed tagged image file (TIF) format [27].

For calibration of the IIF read-out signal, the excitation light intensity level was adjusted employing a recently developed calibration tool based on fluorescent calibration beads [19]. Fluorescent signals of ANA patterns and microbeads were characterized by regional, topological, and texture/surface descriptors by employing image data of DAPI and FITC for cells and FITC as well as Cy5 for beads. A minimum of 50 beads were counted for each parameter analysis. The obtained mean fluorescence intensities (MFI) reflect the specific ANA reactivity of the serum sample. The final read out is expressed as international units (IU) per mL for autoAb to dsDNA in accordance with the international reference material WO80 and in arbitrary units (AU) per mL for the remaining specific autoAb in accordance with internal reference sera.

Statistical analysis

Statistical analysis was performed using MedCalc software (MedCalc®, Mariakerke, Belgium; Version 12.4.0). For normal distribution testing, the Kolmogorov-Smirnov test was applied. Further, the Kruskal-Wallis test was used for testing the difference among defined groups. p-Values below 0.05 were considered to be significant. The cut-off value of single parameters was determined using receiver operating characteristics (ROC) curve analysis. Further, inter-rater agreement (Cohen's κ) and McNemar's test were used for testing the concordance of CytoBead ANA with routine ANA IIF and ELISA.

Correlation of data was analyzed by determining Spearman's rho coefficient of correlation.

Results

Cut-off determination of second generation ANA analysis

In order to determine the cut-offs of the CytoBead ANA for specific autoAb analysis to CENP-B, SS-B/La, Sm, RNP-Sm, dsDNA, Scl-70, SS-A/Ro60, and SS-A/Ro52 a test set of 515 sera consisting of 101 blood donors (BD) and 103 patients with systemic sclerosis (SSc), 46 patients with Sjögren's syndrome (SjS), 174 patients with SLE, 36 patients with rheumatoid arthritis (RA), 34 patients with other SARD, and 21 patients with infections was run on the automated IIF interpretation system Aklides™. Results were subjected to ROC curve analysis. From the ROC curve, cut-off values were determined for all assays to give a diagnostic specificity of at least 95% which correspond to 95.0% specificity showing the following values for autoAb to dsDNA, CENP-B, SS-B/La, Sm, RNP-Sm, Scl-70, SS-A/Ro52, and SS-A/Ro60: 10.0 IU/mL, 9.7 AU/mL, 11.0 AU/mL, 9.4 AU/mL, 8.7 AU/mL, 5.5 AU/mL, 10.2 AU/mL, and 11.5 AU/mL, respectively.

For ANA testing by IIF on HEP-2 cells, the cut-off of 100 MFI was used as determined in an earlier study [25]. Pretesting confirmed the established cut-off value for ANA testing in the present study.

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Table 2: Assay performance of the CytoBead ANA assay.

	CENP-B	La/SS-B	RnP-Sm	Sm	Scl-70	SS-A/Ro52	SS-A/Ro60		dsDNA
Intra-assay variance									
AU/mL	79.3	116.3	130.1	51.8	80.8	117.3	31.7	IU/mL	62.5
SD	7.7	9.7	5.8	7.3	6.4	13.1	4.8	SD	4.6
CV, %	9.7	8.3	4.5	14.1	7.9	11.2	15.1	CV, %	7.3
Inter-assay variance									
AU/mL	76.7	117.6	130.5	50.9	77.8	117.7	30.6	IU/mL	67.5
SD	8.5	11.4	8.5	6.4	6.2	10.7	5.6	SD	8.8
CV, %	11.1	9.7	6.5	12.6	8.0	9.1	18.3	CV, %	12.9

Autoantibodies to dsDNA, SS-B, CENP-B, Sm, RNP-Sm, Scl-70, SS-A/Ro60, and SS-A/Ro52 were determined by CytoBead ANA in reference sera diluted 1–80. Intra-assay CV was determined by eight measurements for each serum while inter-assay CV was assessed by analyzing eight determinations for each serum on three different days. CV, coefficient of variation; MFI, mean fluorescence intensity; SD, standard deviation.

Intra- and inter-assay coefficient of variation analysis

The intra- and inter-assay coefficients of variation (CV) were analyzed using reference sera with the corresponding autoAb specificities. Intra-assay CV was determined by eight measurements for each serum while inter-assay CV was assessed by analyzing eight determinations for each serum on three different days.

The intra-assay CV of the CytoBead ANA assay for autoAb to dsDNA, CENP-B, SS-B/La, RNP-Sm, Sm, Scl-70, SS-A/Ro52, and SS-A/Ro60 ranged from 4.5% to 15.1% and inter-assay CV from 6.5% to 18.3% (Table 2).

ANA and specific antibody analysis by CytoBead ANA

In total, 697 serum samples were analyzed with CytoBead ANA assay for the detection of ANA on HEp-2 cells and specific autoAb to dsDNA, CENP-B, SS-B/La, Sm, RNP-Sm, Scl-70, SS-A/Ro52, and SS-A/Ro60. Antinuclear antibodies assessment revealed significantly different levels in the cohorts investigated (Kruskal-Wallis test, $p < 0.001$, see Supplemental Material, Figure 1 that accompanies the article at <http://www.degruyter.com/view/j/cclm.2015.53.issue-12/cclm-2015-0083/cclm-2015-0083.xml?format=INT>). In patients with SLE, SSc, RA, myositis (MYO), and undifferentiated/mixed connective tissue disease (CTD), ANA testing by CytoBead assay demonstrated prevalences between 88.9% and 100% (Table 3). In contrast, ANA was detected in 20/101 (19.8%) BD and in 10/21 (47.6%) patients with infectious disease (ID). Out of 13 positive ANA findings in patients with autoimmune liver disease (ALD) nine (69.2%) were from patients with AIH.

All specific autoAb demonstrated significantly different levels in the cohorts investigated (Kruskal-Wallis test, $p < 0.001$, respectively, Figure 2). Highest prevalences were determined for autoAb to SS-A/Ro60 (78.3%), SS-A/Ro52 (76.1%) and SS-B/La (37.0%) in patients with SjS, to RNP-Sm (53.8%) in patients with CTD, to CENP-B (53.4%) and Scl-70 (40.8%) in patients with SSc, and to dsDNA (58.0%) and Sm (20.1%) in patients with SLE (Table 3).

Prevalences of SARD-specific autoAb in patients with IBD and ALD as well BD ranged from 0.0% to 3.8%. Two (18.2%) of the 11 patients with PPA demonstrated positive findings for anti-dsDNA autoAb testing only. One of these patients scored positive by ELISA as well. Apart from that, patients with PPA did not demonstrate any positive specific autoAb. The high prevalence of ANA in patients with ID was in part due to high prevalence of low-titer anti-SS-A/Ro52 (42.9%) and anti-RNP-Sm autoAb (33.3%).

With regard to the classical two-tier ANA analysis recommended, ANA negative patients with positive specific autoAb results are of particular concern. In our study cohort, we detected one positive finding for anti-SS-A/Ro60 autoAb and three for anti-SS-A/Ro52 autoAb in 3/266 (1.1%) ANA negative individuals (see Supplemental Material, Table 1). All those three patients suffered from RA. Furthermore, only one ANA-negative patient with SARD who suffered from SjS demonstrated a positive anti-CENP-B autoAb. Classical testing confirmed these results by IIF on HEp-2 cells and anti-SS-A/Ro60, anti-SS-A/Ro52, as well as anti-CENP-B ELISA in the four ANA-negative sera.

Comparison of classical and CytoBead ANA testing

The assay performance of second generation combined ANA testing by CytoBead was further evaluated by

Table 3: Prevalence of antinuclear antibody (ANA) by second generation combined screening and confirmatory ANA testing and classical ANA analysis in 697 patients and controls.

		SSc (n=103), %	SJS (n=46), %	SLE (n=174), %	RA (n=36), %	CTD (n=13), %	MYO (n=21), %	ID (n=21), %	PPA (n=11), %	ALD (n=93), %	IBD (n=78), %	BD (n=101), %
HEp-2 cells	CytoBead	100.0	97.8	100.0	94.4	100.0	95.2	47.6	9.1	13.9	0.0	19.8
	ANA											
La/SS-B	Classical	100.0	97.8	98.9	86.1	100.0	95.2	33.3	9.1	13.9	0.0	16.8
	ANA											
CENP-B	CytoBead	3.9	37.0	17.2	2.8	0.0	0.0	12.9	9.1	0.0	0.0	2.9
	ELISA	3.9	37.0	16.7	2.8	0.0	0.0	9.5	0.0	0.0	0.0	3.8
CENP-B	CytoBead	53.4	19.6	4.0	2.9	0.0	0.0	0.0	0.0	3.2	0.0	0.0
	ELISA	50.5	9.8	2.9	5.5	0.0	0.0	4.8	0.0	3.2	0.0	0.0
Sm	CytoBead	0.0	4.3	20.1	0.0	7.7	9.5	0.0	0.0	0.0	0.0	0.0
	ELISA	0.0	4.3	24.1	0.0	7.7	14.3	0.0	0.0	0.0	0.0	2.9
RNP-Sm	CytoBead	3.9	8.7	29.9	0.0	53.8	33.3	0.0	0.0	0.0	0.0	0.0
	ELISA	3.7	6.5	27.6	0.0	53.8	33.3	0.0	0.0	0.0	0.0	2.9
dsDNA	CytoBead	8.7	10.9	58.0	13.9	15.4	9.5	9.5	18.2	0.0	0.0	2.9
	ELISA	8.7	19.6	62.1	13.9	23.1	9.5	9.5	9.1	0.0	0.0	3.8
Scl-70	CytoBead	40.8	2.2	6.3	5.6	7.7	4.8	4.8	0.0	0.0	0.0	1.9
	ELISA	40.8	2.2	5.7	5.6	7.7	4.8	9.5	0.0	0.0	0.0	2.9
SS-A/Ro60	CytoBead	15.5	78.3	45.4	25.0	0.0	9.5	4.8	0.0	0.0	0.0	3.8
	ELISA	14.6	78.3	43.1	19.4	0.0	9.5	0.0	0.0	0.0	0.0	3.8
SS-A/Ro52	CytoBead	21.4	76.1	33.9	27.8	15.4	42.9	19.0	0.0	3.2	0.0	1.9
	ELISA	25	76.1	30.5	27.8	15.4	42.9	19.0	0.0	3.2	0.0	0.0

Antinuclear antibodies (ANA) and autoantibodies to dsDNA, SS-B, CENP-B, Sm, RNP-Sm, Scl-70, SS-A/Ro60, and SS-A/Ro52 were determined by classical indirect immunofluorescence (IIF) and enzyme-linked immunosorbent assay (ELISA), respectively. Further, ANA and specific autoantibodies were analyzed by second generation ANA testing (CytoBead) including simultaneous ANA analysis on HEp-2 cells and specific autoantibody determination. ALD, autoimmune liver disease; BD, blood donors, CTD, undifferentiated and mixed connective tissue disease; IBD, inflammatory bowel disease; ID, infectious disease; MYO, myositis; PPA, paraproteinemia; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; SjS, Sjögren's syndrome; SSc, systemic sclerosis.

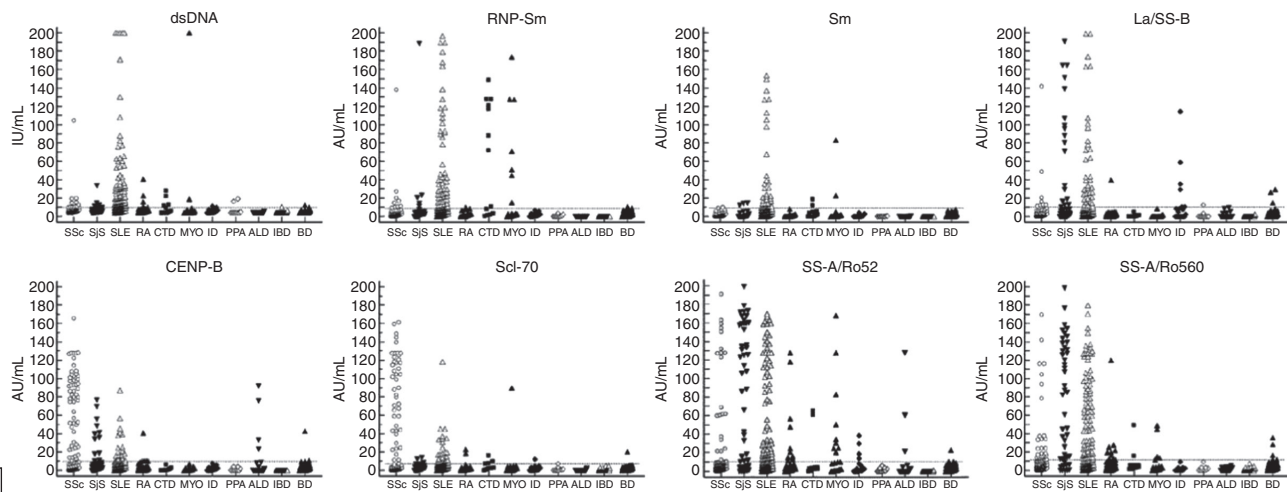


Figure 2: Specific autoantibody (autoAb) testing to dsDNA, SS-B, CENP-B, Sm, RNP-Sm, Scl-70, SS-A/Ro60, and SS-A/Ro52 by CytoBead ANA employing indirect immunofluorescence in 697 patients and controls.

Cut-offs for the determination of specific autoAb are indicated as dotted line. ALD, autoimmune liver disease; BD, blood donors; CTD, undifferentiated and mixed connective tissue disease; IBD, inflammatory bowel disease; ID, infectious disease; MYO, myositis; PPA, paraproteinemia; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; SjS, Sjögren's syndrome; SSc, systemic sclerosis.

comparison to conventional autoAb analysis. Thus, 697 serum samples were tested for classical ANA by an IIF assay on HEp-2 cells and for specific autoAb by ELISA. Data obtained were compared to those analyzed with CytoBead ANA, respectively (Table 4). The CytoBead ANA revealed 428 (61.4%) positive ANA findings and 360 (84.1%) of those ANA-positive samples demonstrated at least one positive specific autoAb by this test. Classical ANA testing detected 420 positives (60.3%) and of those samples 357 (85.0%) showed at least one positive specific autoAb by ELISA.

The agreement between ANA detected by conventional IIF and CytoBead ANA on HEp-2 cells was very good ($\kappa > 0.95$). As a matter of fact, there was also no significant difference of both approaches in accordance with McNemar's test ($p > 0.08$). CytoBead ANA demonstrated a higher yet not significant prevalence of ANA positive findings in the whole study cohort in comparison with classical IIF on HEp-2 cells (434/697, 62.3% vs. 422/697, 60.5%; $p > 0.54$).

Regarding the comparison of specific autoAb determined by ELISA and CytoBead ANA, agreement was very good ($\kappa > 0.80$) except for anti-Sm autoAb ($\kappa = 0.77$). The respective ELISA revealed a higher yet not significant prevalence for this autoAb in patients with SLE (ELISA 42/174, 24.1% vs. CytoBead ANA 35/174, 20.1%; Table 3).

The differences of both methods for the analysis of autoAb to SS-B/La, RNP-Sm, Scl-70, SS-A/Ro60, and SS-A/Ro52 were not significant (McNemar, $p = 0.63, 0.45, 1.00, 0.17, 0.06$, respectively).

Discrepant results by both techniques are displayed in Supplemental Material, Figure 2. In total, 103 samples demonstrated discrepant results by CytoBead and ELISA. In the great majority of those cases (71/103, 68.9%, $p < 0.001$) the CytoBead result was confirmed by a third method [line immunoassay (LIA)]. Thus, seven of the 13 individuals with anti-SS-A/Ro60 positivity by CytoBead ANA only were patients suffering from SLE whose anti-SS-A/Ro60 positivity was confirmed by LIA. In contrast, only one patient out of six anti-SS-A/Ro60 autoAb positive individuals by ELISA suffered from SLE. However, all six ELISA positive individuals were tested negative for anti-SS-A/Ro60 autoAb by LIA.

The five anti-RNP-Sm positive patients by CytoBead ANA only comprise four patients with SLE (2 confirmed by LIA) and one with SjS. Notably, the ELISA revealed just two anti-RNP-Sm positive BD which were not confirmed by both CytoBead ANA and LIA.

In contrast, the determination of autoAb to dsDNA, CENP-B, and Sm demonstrated significant differences for the whole study set (2.4%, 1.3%, 1.8%; $p = 0.004, 0.030, 0.012$, respectively). The ELISA for the detection of anti-dsDNA autoAb revealed a higher yet not significant prevalence in patients with SLE, SjS, and CTD. In the control groups comprising patients with PPA, ALD and IBD as well as BD, ELISA and CytoBead detected an equal number of anti-dsDNA autoAb positives (7/304, 2.3%). In total, there were 31 discrepant results for anti-dsDNA autoAb testing (Supplemental Material, Table 2). The CytoBead ANA revealed three anti-dsDNA autoAb positive patients

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Table 4: Comparison of second generation combined antinuclear antibody (ANA) screening and confirmatory testing with classical ANA analysis.

CytoBead ANA	Classical IIF		Cohen's κ^a	95% CI	Difference, %	95% CI	p ^b -Value
	Pos	Neg					
ANA							
Pos	416	12	0.95	0.93–0.98	1.15	–0.11% to 1.96%	0.077
Neg	4	265					
CytoBead ANA	Classical ELISA						
	Pos	Neg					
SS-B/La							
Pos	56	3	0.96	0.93–0.99	0.29	–0.36 to 0.58	0.630
Neg	1	623					
CENP-B							
Pos	65	10	0.91	0.85–0.96	1.28	0.06–1.84	0.039
Neg	2	547					
Sm							
Pos	36	4	0.77	0.67–0.87	1.83	0.39–2.70	0.012
Neg	16	601					
RNP-Sm							
Pos	70	5	0.95	0.91–0.99	0.45	–0.44 to 0.98	0.450
Neg	2	583					
dsDNA							
Pos	125	7	0.86	0.82–0.91	2.45	0.79–3.60	0.004
Neg	24	539					
Scl-70							
Pos	57	2	0.95	0.91–0.99	0.15	–0.53 to 0.67	1.000
Neg	3	603					
SS-A/Ro60							
Pos	136	13	0.92	0.88–0.95	1.06	–0.38 to 2.14	0.167
Neg	6	508					
SS-A/Ro52							
Pos	135	5	0.96	0.94–0.99	0.73	–0.03 to 0.73	0.060
Neg	0	546					

Antinuclear antibodies (ANA) and autoantibodies (autoAb) to dsDNA, SS-B, CENP-B, Sm, RNP-Sm, Scl-70, SS-A/Ro60, and SS-A/Ro52 were determined by classical indirect immunofluorescence (IIF) and enzyme-linked immunosorbent assay (ELISA), respectively. Data were compared with second generation ANA testing including simultaneous ANA analysis on HEp-2 cells and specific autoAb determination by CytoBead ANA. ^aCohen's κ ($\kappa \leq 0.2$ poor; 0.21–0.40 fair; 0.41–0.6 moderate; 0.61–0.8 good; 0.81–1.0 very good agreement); ^bMcNemar test.

with SLE which scored negative by ELISA and were confirmed by LIA. Regarding controls, there were just two false-positive anti-dsDNA autoAb patients with ID and PPA not confirmed by LIA. In contrast, 10 patients with SLE demonstrated a positive anti-dsDNA autoAb by ELISA which were not confirmed by CytoBead ANA. Five of those 10 ELISA positives were also negative for anti-dsDNA autoAb by LIA. Furthermore, there were five BD and one patient with ID positive for anti-dsDNA autoAb by ELISA only. As a matter of fact, the areas under the curve (AUC) obtained by ROC curve analysis with SLE as disease group and all other cohorts as control group did not reveal a significant difference for both anti-dsDNA detection methods (AUC ELISA: 0.874 vs. AUC CytoBead: 0.836, $p > 0.05$).

The differences in the prevalence of anti-Sm and anti-CENP-B autoAb were also not significant in the study cohorts. The ELISA revealed 11 anti-Sm autoAb positive patients with SLE which were found negative by CytoBead ANA. Ten out of those 11 patients demonstrated a homogenous ANA pattern by IIF and were anti-dsDNA autoAb positive by CytoBead ANA. Thus, only one SLE patient was to be missed by CytoBead ANA testing based on anti-dsDNA and anti-Sm antibodies. Of interest, the inability to detect anti-Sm autoAb was not limited to CytoBead ANA, as five of those 11 SLE patients were also anti-Sm autoAb positive by LIA. Additionally, there were four BD and one patient with MYO scoring positive for anti-Sm autoAb by ELISA only and confirmed

neither by CytoBead ANA nor LIA. In contrast, the CytoBead ANA revealed four patients with SLE positive for anti-Sm autoAb which were all confirmed by LIA but not by ELISA. Notably, there were no further discrepant anti-Sm autoAb positives by CytoBead ANA in any other cohort including controls.

As a matter of fact, all CytoBead ANA anti-CENP-B autoAb results for patients and controls discrepant by ELISA were confirmed by LIA (Supplemental Table 2).

Based on the quantitative data obtained for specific autoAbs by classical ELISA and CytoBead assay, Spearman's rho coefficients of correlation were determined ranging from 0.30 to 0.64 (anti-dsDNA: 0.64, anti-CENP-B: 0.49, SS-B/La: 0.30, anti-Sm: 0.38, RNP-Sm: 0.44, anti-Scl-70: 0.30, anti-SS-A/Ro52: 0.52, anti-SS-A/Ro60: 0.44; $p < 0.0001$, respectively).

Discussion

Since its first description in 1957, ANA testing by IIF remains the “gold” standard technique for the serological diagnosis of SARD [28, 29]. Recently, ANA detection has been confirmed as the first level test for the serology of SARD by an international expert panel [1]. As a matter of fact, classical ANA testing requires the characterization of positive ANA findings by immunoassays for the detection of specific autoAb to nuclear and cytoplasmic autoantigenic targets in the framework of the recommended two-tier approach [30–32]. However, this diagnostic two-step process has proved to be laborious and created constraints in particular for larger laboratories with high throughput [16, 30]. Several attempts have been made to replace the two-tier testing by one analysis deploying different assay platforms [3, 15, 30, 33–35]. Noteworthy, these new multiplex techniques including addressable laser bead immunoassays and several fully automated closed systems do not appear to reach the diagnostic sensitivity of ANA testing by IIF using HEp-2 cells and resulted even in misdiagnosis of SARD [3, 7, 36].

Thus, digital fluorescence paving the way for the development of novel pattern recognition algorithms for automated standardized autoAb IIF reading has provided new perspectives for appropriate ANA analysis [13, 37–39]. Several automated fluorescent interpretation systems have been developed recently and first reports seem to support their usefulness in routine autoimmune diagnosis [3, 25, 26, 40–45]. For the first time in the history of ANA analysis, this intriguing new technology creates the basis for the combination of screening and corresponding

confirmatory testing. A similar approach as has been demonstrated for efficient automated antineutrophil cytoplasmic antibody testing recently [46].

Thus, to the best of our knowledge, we established the first one-step quantitative multiplex ANA test deploying HEp-2 cells for ANA screening and autoantigen-coated fluorescent microparticles for the simultaneous quantitative detection of specific autoAb.

Analysis of assay performance data revealed acceptable results for the detection of ANA and specific autoAb by the CytoBead technology and they are in line with corresponding data of other immunoassays reported elsewhere [3, 21, 25]. Concerns must be raised, though, as we cannot exclude that the assay performance data obtained for specific autoAb testing by CytoBead ANA could be biased due to the overlap of the test set with the study set. On the strength of our past experience, however, we have not noticed such bias for ANCA analysis by the CytoBead technology using a similar approach [46, 47].

The use of digital fluorescence provided the basis for a quantitative analysis not only of specific autoAb but also ANA that can be standardized by calibrated interpretation systems [19]. This ushers in a new age of standardization of ANA testing which was not feasible with classical ANA testing by IIF.

The obtained diagnostic sensitivity of ANA for SARD confirmed the high sensitivity and, thus, high negative predictive value of IIF on HEp-2 cells for this autoAb assessment [7]. In contrast, the diagnostic specificity was moderate in particular due to false-positive findings in patients with ID and even BD. However, this confirms the concept of the recommended two-tier ANA analysis for the serological diagnosis of SARD [1]. In patients with ALD, the majority of positive ANA findings 9/13 (69.2%) was determined in patients with AIH. This corresponds to a diagnostic sensitivity of ANA in AIH of 45.0% and is in agreement with the fact that ANA is one of the classification criteria of AIH [6].

The agreement of ANA and specific autoAb findings by second generation testing with conventional techniques, such as IIF and ELISA, ranged from good to very good in this study. However, the comparative analysis of several single markers like anti-dsDNA autoAb demonstrated a significant difference between classical and combined ANA testing. In the case of anti-dsDNA autoAb assessment, the novel CytoBead ANA detected fewer positives in patient and controls cohorts but the differences were not significant. These differences can be due to the different autoantigenic epitope presentation on the respective solid phases employed in ELISA and CytoBead. Autoantigens used for CytoBead assessment are covalently bound to the

chemically activated surface of fluorescent microbeads [21]. In ELISA, autoantigens are immobilized by adhesion only and in particular in the case of dsDNA can lead to a higher rate of false-positive results in comparison with the isotopic Farr assay or *Crithidia luciliae* immunofluorescence test [48]. In support of the thesis that antigen presentation is the most likely cause for discrepant results stems from the fact that the CytoBead assay fails to identify ELISA positive results (false negative), as well as detects ELISA negative tests (false negative).

Combined second generation ANA screening and confirmatory testing addresses another shortcoming of the classical two-tier ANA analysis. False-negative ANA findings by IIF on HEp-2 cells have been reported particularly for anti-SS-A/Ro autoAb-positive patients suffering from SARD [2, 17]. Simultaneous detection of ANA and specific autoAb, such as SS-A/Ro, can minimize this risk substantially and further increase the already high negative predictive value of ANA testing. In our study, we detected four (1.5%) ANA-negative patients with positive anti-SS-A or anti-CENP-B autoAb by second generation ANA testing. These patients suffering from RA and SjS would have been missed with the two-tier approach as ANA negativity and positivity for anti-SS-A and anti-CENP-B autoAb were confirmed by classical testing in this study. Remarkably, Bossuyt and Luyckx reported in 1840 consecutive ANA-negative samples 11 (0.6%) anti-SS-A autoAb positive patients [2], although fixation techniques and thus the sensitivity of ANA testing might have improved lately. Two of those 11 patients suffered from RA like three in our study. A further four of the 11 ANA-negative and anti-SS-A autoAb-positive patients were diagnosed with SARD, such as SLE and SjS. In contrast to second generation ANA testing, classical two-tier ANA analysis could have missed these samples and brought about misdiagnosis of patients.

Conclusions

The good concordance of this comparative clinical analysis supports the assumption that the novel combined reaction IIF environment for one-step ANA analysis employing HEp-2 cells and autoantigen-coated fluorescent beads as respective targets can provide at least the same assay performance like classical two-tier ANA testing. Thus, this new approach referred to as second generation ANA testing can meet the demand of modern routine service laboratories for the serology of SARD by addressing disadvantages of the currently recommended two-tier ANA analysis.

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