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Original source: Barrale M, Bizzaro N, Villalta D, Brusca I. Profilo autoanticorpale su microblot array nelle malattie autoimmuni sistemiche: efficienza diagnostica in confronto con il metodo di immunofluorescenza indiretta su cellule HEp-2. *La Rivista Italiana della Medicina di Laboratorio* 2026;22:000–000.

DOI: 10.23736/S1825-859X.26.00327-0 · © 2026 Edizioni Minerva Medica · Article type: Original Article

Autoantibody profiling on Microblot Array in systemic autoimmune diseases: diagnostic efficiency compared with the indirect immunofluorescence method on HEp-2 cells

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Abstract

Background: Indirect immunofluorescence (IIF) on HEp-2 cells is still considered the gold standard for screening for antinuclear antibodies (ANA) in systemic autoimmune rheumatic diseases. However, this method has several limitations, including operator-dependent interpretation, limited standardization, and the need for additional tests to identify specific autoantibody targets. Multiplex immunometric technologies that allow the simultaneous detection of multiple autoantibodies are emerging as promising tools for more comprehensive serological characterization.

Methods: We evaluated the diagnostic performance of a multiplex microblot array (MBA) capable of detecting 43 autoantibody specificities associated with systemic autoimmune diseases. A total of 382 samples were analyzed, including 152 patients with systemic autoimmune diseases and 230 control subjects. The results obtained with the MBA method were compared with ANA by IIF on HEp-2 cells.

Results: The diagnostic sensitivity of the MBA method was 98%, and that of the IIF method was 89.5%. The overall specificity was 75.7% for MBA and 78.3% for IIF. When evaluated individually, all antibodies included in the MBA panel showed very high diagnostic specificity, with a mean value of 99%. The overall concordance between MBA and ANA-IIF was 71.4%, with a mean Cohen's κ coefficient of 0.450.

Conclusions: The multiplex microblot array showed moderate concordance with ANA-IIF, while still allowing highly specific identification of individual target autoantigens. The broad antigenic coverage allows for rapid and simultaneous characterization of complex autoantibody profiles and may represent a useful complementary tool within diagnostic algorithms for systemic autoimmune diseases.

Key words: *Antinuclear antibodies; Immunoassay; Indirect fluorescent antibody technique; Autoantibodies; Autoimmune diseases.*

Introduction

Autoimmune rheumatic diseases (ARD) — including systemic lupus erythematosus, systemic sclerosis, Sjögren's syndrome, mixed connective tissue disease, and idiopathic inflammatory myopathies — are characterized by the presence of circulating autoantibodies directed against a broad range of nuclear and cytoplasmic antigens.

Among these biomarkers, anti-cellular antigen antibodies (ANA) play a central role in the diagnosis and classification of ARD. The determination of ANA by indirect immunofluorescence (IIF) on HEp-2 cells has long been considered the reference method for screening and is recommended by the main national and international guidelines. The ANA-IIF test indeed has high analytical sensitivity owing to its ability to detect a broad spectrum of autoantibodies (about a hundred) directed against numerous cellular structures. It also has some limitations, however. In particular, interpretation of the fluorescence patterns depends strongly on operator experience, resulting in considerable intra- and inter-laboratory variability. Moreover, a positive ANA-IIF screening result does not allow direct identification of the antigenic target of the autoantibodies, making additional tests necessary for the detection of specific anti-nucleocytoplasmic antigen antibodies.

In recent years, automated multiparametric immunometric technologies have been developed with the aim of overcoming these limitations. These systems allow the simultaneous detection of multiple antigen-specific autoantibodies in a single analysis, offering greater standardization and diagnostic efficiency. Among these, microblot array technologies combine the principles of immunoblot assays with multiplex detection and probably represent the antibody detection techniques that will be widely used in the future.

The aim of this study was to evaluate the diagnostic performance and analytical reliability of a new multiplex microblot array and to compare its results with those obtained by conventional determination of ANA by IIF on HEp-2 cells, in order to verify whether this new method has higher diagnostic sensitivity and specificity than the IIF HEp-2 method — that is, whether it is able to replace it in ANA screening.

Materials and methods

This observational study included 382 serum samples, of which 152 were from patients with ARD and 230 from subjects with viral infections or affected by various non-autoimmune diseases, serving as the control group. The distribution of the samples is shown in Table I.

The tests analyzed in the study had been requested as part of routine diagnostic activity and were analyzed in anonymized and de-identified form prior to analysis, in accordance with the Declaration of Helsinki and Italian legislation (Authorization of the Data Protection Authority no. 9 of 12 December 2013).

Table I. — Diagnosis of patients and control subjects.

Systemic autoimmune disease group	N.	Control group	N.
Systemic lupus erythematosus	33	Viral infections (CMV, HCV, HBV, EBV)	77
Mixed connective tissue disease	20	Patients hospitalized for non-autoimmune conditions	107
Autoimmune myositis	20	Various non-autoimmune diseases	23
Sjögren's syndrome	29	Fever of unknown origin	23
Systemic sclerosis	50		
Total	**152**		**230**

ANA determination was performed by IIF on HEp-2 cells at an initial dilution of 1:80, using the automated QUANTA LITE system (Inova Diagnostics, San Diego, CA, USA). Positive samples were subsequently titrated up to a dilution of 1:1280. A titer ≥1:80 was considered indicative of ANA positivity.

Detection of the individual autoantibody specificities was performed using a microblot array (MBA) system (BioVendor/TestLine, Brno, Czech Republic). The test evaluated in this study consists of a panel of both disease-specific and disease-associated markers relating to various systemic autoimmune diseases, which includes the following 43 nuclear and cytoplasmic autoantigens: dsDNA, nucleosomes, histones, SmB, SmD, RNP-A, RNP-68/70, RNP-C, Ro60, Ro52, La, ribosomal P, PCNA, Ku, PM/Scl-100, PM/Scl-75, CENP-A, CENP-B, Th/To, RNA polymerase III, Scl70, fibrillarin, NOR90, Mi-2, TIF1γ, KS, YARS, ZoA, ZoB, MDA5, NXP2, SAE1, SAE2, SRP54, PL-7, PL-12, EJ, OJ, Jo1, PDGFR-β, nucleolin, DFS70, and AMA-M2.

The assay also includes internal controls and a four-point calibration profile to ensure the analytical validity of the test and the quantification of antibody concentration.

Statistical analysis

Concordance between MBA and ANA-IIF was assessed using overall concordance and Cohen's kappa coefficient (κ). The overall diagnostic specificity of the two methods compared, and that of each individual antibody for the MBA method, was calculated using the control population. The area under the curve was calculated using ROC curve analysis.

Results

In the 152 patients with systemic autoimmune disease, the diagnostic sensitivity of the MBA method was 98% and that of the IIF method was 89.5%. This difference in sensitivity is mainly due to the autoimmune myositis group, where only eight out of 20 samples (40%) were positive by IIF versus 20/20 (100%) by MBA. The MBA method predominantly yielded multiple positivities:

where the result coincided with that expected for the disease, antibody levels were very high, whereas low-concentration positivities — mostly just above the cutoff — mostly concerned antibodies generally not associated with the disease under examination.

When evaluated individually, all the antibodies included in the MBA panel showed very high diagnostic specificity, with a mean value of 99% (Table II). Evaluated overall, the specificity derived from the sum of all antibodies present in the MBA across the 230 control samples was 75.7%, compared with 78.3% for IIF at a dilution of 1:80. At a dilution of 1:160, the specificity of IIF was 88.2%.

The overall concordance between MBA and IIF was 71.4%, with a mean Cohen's κ coefficient of 0.450. Discrepant results were found predominantly in viral infections (greater positivity with MBA) and in non-autoimmune diseases (greater positivity with ANA-IIF). The autoantibodies most frequently responsible for false-positive results with MBA were anti-Ro60, anti-RNP-A, and anti-nucleosome (Table III).

The overall diagnostic efficiency — that is, the ability to correctly classify both true-positive and true-negative samples — was 84.5% for MBA and 82.7% for IIF. The area under the ROC curves was entirely comparable (0.876 for MBA and 0.887 for IIF).

Table II. — Diagnostic specificity of the microblot array for the individual autoantibodies included in the profile.

Autoantibody	Diagnostic specificity
dsDNA	97.3%
Nucleosome	96.0%
Histones	98.2%
SmB	99.7%
SmD	99.1%
RNP-A	96.0%
RNP-70	98.7%
RNP-C	98.2%
Ro60	91.5%
Ro52	97.3%
La	100%
Ribosomal P	100%
PCNA	99.6%
Ku	99.1%
PM-Scl100	100%
PM-Scl75	99.6%
Th/To	100%
RNA polymerase III	100%
Scl-70 (Topoisomerase I)	99.6%

Autoantibody	Diagnostic specificity
CENP-A	99.6%
CENP-B	99.7%
Fibrillarin	100%
NOR90	99.1%
Mi-2	98.7%
Jo-1	100%
TIF1γ	99.1%
MDA5	99.6%
NXP2	99.1%
SAE1	100%
SAE2	99.6%
SRP54	98.7%
PL-7	100%
PL-12	100%
EJ	99.1%
OJ	100%
KS	100%
YARS	99.6%
ZoA	100%
ZoB	100%
PDGFR-β	100%
Nucleolin	100%
AMA-M2	100%

Mean diagnostic specificity: 99.0%.

Table III. — Main autoantibodies associated with the false-positive results observed in the control group with the MBA method.

Autoantibody specificity	N.	Percentage of false positives
Anti-Ro60	19	33.9%
Anti-RNP-A	7	12.5%
Anti-nucleosome	7	12.5%
Anti-Ro52	6	10.7%
Anti-dsDNA	4	7.1%
Anti-histone	4	7.1%
Other specificities	9	16.1%
Total	**56**	**100.0%**

Discussion

IIF on HEp-2 cells is considered the reference method for ANA screening, but its undeniable limitations have over the years prompted the biomedical industry in this field to develop, first, automated systems for the preparation and reading of immunofluorescence preparations and, subsequently, multiparametric analytical methods that could provide greater diagnostic specificity and lower analytical variability. In particular, these technologies allow the simultaneous detection of numerous autoantibodies and make it possible to obtain complete serological profiles in a single analysis. In this sense, multiplex immunometric assays have emerged as promising alternatives to IIF, capable of improving analytical standardization and laboratory efficiency in the screening phase. In this study we evaluated the diagnostic performance of a new multiplex system based on microblot array technology for the detection of a broad panel of autoantibodies, comparing it with that of the conventional ANA-IIF method.

The overall concordance between MBA and ANA-IIF was moderate, at 71.4%. The discrepancies observed between the two methods are probably attributable to the different analytical principles on which they are based. Whereas ANA-IIF detects an overall reactivity against cellular structures, multiplex assays identify antibodies directed against predefined antigen panels.

The greater diagnostic sensitivity of the MBA method compared with IIF (98% vs. 89.5%) clearly indicates that the panel of 43 antigens is adequate and sufficient to detect all the main antibodies associated with ARD. This was expected from the data on myositis, where the broad antigenic profile of the MBA method recorded no false-negative results, whereas the IIF method was negative in 60% of the samples. As for specificity, both methods in this study are around 76–78%. If one considers the 1:160 dilution — which is the clinically significant decision level — the specificity of IIF increases by 10 percentage points to around 88%, but sensitivity inevitably falls to 71%.

False positives with MBA in the control group were mainly associated with anti-Ro60 autoantibodies in the serum of subjects with viral infections. The presence of such autoantibodies in non-autoimmune conditions has been previously described and may be due to transient polyclonal activation associated with viral infection.

A previous study of ours that evaluated a multiparametric system using particle-based multi-analyte technology (PMAT) and including 29 antigens had demonstrated a sensitivity of 83% and a specificity of 78%. Although the two studies are not directly comparable because of the different patient series studied, it is likely that the greater sensitivity of the MBA method compared with PMAT may be due to the larger number of antigens (43 versus 29). Specificity, on the other hand, is entirely comparable (76% MBA vs. 78% PMAT). In this regard, it is extremely interesting to note that the more the number of antigens included in the assays increases, the closer one gets to the performance of the IIF method — that is, the specificity of the multiparametric system falls to the same levels as the IIF method. Indeed, when a multiparametric system is used, even if the specificity of each individual antibody is 99%, when the overall specificity of the system is calculated the proportion of false positives for each antibody adds up and therefore takes on increasingly lower values compared with that of the individual antibody. This particular aspect of multiparametric systems leads us to consider that the ideal would be to configure multiparametric

systems in such a way that the specificity of the individual antibodies is always 100%, even at the cost of losing a few percentage points in sensitivity.

This study has several strengths: it is the first study to report diagnostic accuracy data for the new MBA method, which uses a very extensive multiparametric profile comprising 43 autoantigens, and it includes a direct comparison with the reference method ANA-IIF. A possible limitation is the monocentric design, which could limit the generalizability of the results.

The integration of these technologies into laboratory diagnostic algorithms may help to reduce subjectivity in the interpretation of ANA and improve the overall efficiency of the laboratory. From a clinical point of view, multiplex profiling could allow more rapid and complete identification of disease-specific autoantibodies, contributing to the definition of molecular subgroups associated with different clinical features.

In conclusion, the multiplex microblot array evaluated in this study has demonstrated that it can already be used within the diagnostic algorithm as a second-level test to identify the antibody specificities responsible for the positivity of screening tests. Its broad antigenic coverage indeed allows complete autoantibody profiling and could represent a valuable complementary tool within the diagnostic algorithms of systemic autoimmune diseases.

Were it to be used as a screening test for ANA, it provides performance equal to or slightly higher than that of the IIF HEp-2 method. Further studies will nevertheless be necessary to confirm the role of multiplex autoantibody assays in routine clinical practice and their correct positioning within the diagnostic algorithm of autoimmune rheumatic diseases.

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Article information

Conflicts of interest. The authors declare that they have no conflicts of interest with any company related to the content of the manuscript.

Studies involving humans and animals. All procedures described in the study and involving human beings were carried out in accordance with the ethical standards established by the Declaration of Helsinki of 1964 and its subsequent amendments.

Informed consent. Informed consent was obtained from all patients included in the study.

History. Published online: —. Accepted: 14 April 2026. Received: 23 March 2026.