Reading and interpretation of ANA immunofluorescence tests: results of a comparative study with six automated systems

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Introduction

Indirect immunofluorescence (IIF) plays an important role in immunological assays for detecting and measuring autoantibodies. However, the method is burdened by some unfavorable features: the need for expert morphologists, the subjectivity of interpretation, and a low degree of standardization and automation [1]. Following the recent statement by the American College of Rheumatology [2] that the IIF technique should be considered as the standard screening method for the detection of anti-nuclear antibodies (ANA), the biomedical industry has developed technological solutions which might significantly improve automation of the procedure, not only in the preparation of substrates and slides, but also in microscope reading. This innovation is based on the principle of digitalization of fluoroscopic images, as an example of computer-assisted diagnosis, and on the classification of patterns using standardized approach (automated positive/negative screening and pattern interpretation) [3-6].

The expected advantages of automated IIF are the reduction of intra- and inter-laboratory variability, the improvement of correlation of staining patterns with corresponding autoantibody reactivities, and higher throughput in laboratory workflow.

Automation of the IIF method can be used for a cost-effective and accurate screening for diagnostically relevant autoantibodies; this technology may play down errors and problems caused by subjective image evaluation and low expertise. In addition, one possible advantage of this new technique lies in measuring fluorescence intensity for quantitative evaluation of antibody concentration, thus avoiding the need for titration.

Currently, several commercial systems are available and have been evaluated in preliminary experimental studies on single devices [7-19] with the purpose of the assessment of the reliability of automated IIF analysis as a standardized alternative for the conventional manual visual approach. Therefore, at present there are not studies comparing the different commercial technological platforms for automated ANA-IIF.

This study was undertaken to verify the level of accuracy of new automatic systems for the reading of ANA samples, specifically in discriminating between ANA-positive and ANA-negative samples. As a second objective, we analyzed the accuracy of these systems in pattern recognition, and checked whether there

is correlation between levels of the analytic signal provided by the instruments and the titer obtained with manual IIF.

Materials and methods

Patients and sera

We collected 105 ANA-positive sera and 40 ANA-negative sera. The preliminary selection of ANA-positive sera was made in eight laboratories of the Study Group on Autoimmune Diseases of the Italian Society of Laboratory Medicine (SIMeL) based on five main criteria: a) the source of sera (sera should be obtained from patients with a confirmed clinical diagnosis of autoimmune disease according to internationally accepted criteria); b) the type of pattern (in order to have a representative number of samples for each of the 15-20 most frequent or clinically more relevant patterns, in accordance with the international nomenclature [20]); c) the presence of a single pattern (i.e. sera with mixed pattern were excluded); d) range of antibody titer (that is, wide range within the same pattern); d) antibody specificity defined by methods other than IIF (ELISA, LIA, blot).

Tables 1 and 2 describe the clinical diagnosis of patients, the IIF pattern and the antibody specificity of the test sera.

Sera identified with acronyms (to anonymize patients) came from the residue of serum samples referred for ANA testing. The 40 ANA-negative sera were obtained from healthy blood donors. The sera were centralized into a single laboratory where the source identification code was recorded and the samples were randomized and renumbered progressively. Eight aliquots of each serum were prepared.

Certification of pattern and titer

Since the assignment of ANA titer and pattern was subject to variables related to the characteristics of the substrate (HEp-2 cells) and the subjectivity of interpretation of operators who selected the candidate sera, a preliminary certification was performed in the following way: one aliquot of each serum, without information about pattern and titer, was sent to each of the six laboratories of our group. Each laboratory tested the sera with the IIF manual method provided to it by one of the six manufacturers of automatic systems. The results were tabulated by recording data in terms of positivity/negativity, pattern and titer (all serum samples were titrated with a dilution of 1: 80 to 1: 1,280. Sera with higher titer were recorded as >1:1,280).

The final assignment of result (pos/neg), of pattern and titer was made by consensus at a meeting held on November 11 and 12, 2012 in Udine (Italy) which was attended by all eight members of the research team. Result was assigned if consensus for pos/neg was reached by at least four of six certifiers, while for the pattern and for the titer, the value observed with higher frequency (mode) was adopted. Seventeen ANA-positive sera and six ANA-negative sera were excluded: for these, there was not a sufficient consensus value in assigning the result of positivity/negativity. Therefore, the final study with automatic instrumentation was conducted on 92 ANA-positive sera and on 34 ANA-negative sera.

Systems for automated ANA screening

Six companies that produce or distribute automatic systems for the reading of ANA-IIF (*EUROPattern,* Euroimmun *AG*, Luebeck, Germany; *Aklides,* Medipan, Dahlewitz, Germany; *NOVA View,* Inova Diagnostics

Inc., San Diego, USA; *Helios,* Aesku Diagnostics, Wendelsheim, Germany; *Zenit G-Sight (I-Sight IFA),* A. Menarini Diagnostics, Florence, Italy; *Image Navigator,* ImmunoConcepts, Sacramento, CA) agreed to participate in the study and placed their system at two laboratories of our group. To ensure the highest accuracy in the production of analytical results, sera were tested with their automated system by specialists of each company, using the following substrates: HEp-2 cells for Euroimmun, Inova and Aesku Diagnostics; HEp-2000 cells for ImmunoConcepts; HEp-2 Zenit IMMCO for Menarini; and HEp-2 Generic Assays for Medipan/Aklides. For all systems, the dilution of sera for the automatic analysis was 1: 80.

Statistical analysis

Statistical analysis was performed by calculating the value of sensitivity and specificity of each of the six systems at the cutoff value suggested by the manufacturers. The accuracy in the detection of patterns and antibody concentration (the intensity of the light signal detected by instrumentation) was evaluated in relation to the pattern and the titer assigned by consensus in the preliminary phase. Finally, to allow a precise comparison between automatic methods and to calculate the specificity in relation to a single value of sensitivity, the light intensity value of the test systems was analyzed and compared by Receiver Operating Characteristics (ROC) curve analysis, with the exclusion of the Helios system which does not provide a value for the sample fluorescence signal.

Results

Positive/negative discrimination of ANA by automated interpretation

Sensitivity (measured on the 92 ANA-positive sera) and specificity (measured on the 34 ANA-negative sera) of the six systems at the cutoff adopted by the manufacturers are described in table 3. Overall sensitivity was 96.6% and overall specificity was 89.2%.

With respect to the ANA pattern, most false negatives were recorded for cytoplasmic patterns, whereas among nuclear patterns those with a low level of fluorescence (ie, multiple nuclear dots, midbody, nuclear rim) were sometimes missed.

Specificity of the six systems at identical values of sensitivity as calculated by ROC curve analysis is shown in table 4.

ANA IIF pattern recognition by automated interpretation

Of the six systems analysed, four (EuroPattern, Aklides, Nova View and G-Sight) were able to provide an interpretation of ANA pattern (although only homogeneous, speckled, nucleolar, centromere, multiple nuclear dots and cytoplasmic patterns could be interpreted). EuroPattern correctly identified ANA patterns in 79% of cases, Aklides in 50%, Nova View in 54%, and G-Sight in 63%.

Quantitative measurement of the light intensity signal

In general, the intensity values of the light signal of various instruments show a good correlation with the titer obtained by manual reading (Spearman's rho between 0.665 and 0.828; $P < 0.001$ for all the systems). However, high or low intensity values do not always coincide with high or low titer values; and vice versa, samples with different titers can show identical signal intensity. Quantitative analysis of the analytic signal carried out separately for each pattern does not improve the correlation between signal intensity and titer.

Discussion

During the last 15 years, the progressive increase of ANA test requests and volume of assays performed in clinical laboratories produced alternative solutions to the ANA-IIF test based on manual or automated monoplex and multiplex immunometric assays (EIA, FIA, LIA), but literature reports demonstrated that these procedures do not provide the same analytical accuracy [reviewed in 21].

The need for standardization of ANA testing brought the biomedical industry to propose technological solutions which may improve the automation of the IIF procedure, not only in the preparation of substrates and slides, but also in microscope reading.

A review of the literature revealed that at least 12 studies have been performed to date on the performance of single automated instruments for ANA reading [7-19]. Cumulative data obtained in more than 7,700 patients showed a positive/negative agreement with the manual IIF method ranging from 0.92 to 0.99 (Table 5).

This study which is the first to compare the diagnostic accuracy of six systems for automated ANA-IIF reading on the same series of sera, showed that all systems are able to perform very well the task for which they were created. Indeed, cumulative automatic discrimination between positive and negative samples had 95% accuracy. Since all companies request that after the instrumental reading, data (or images) are validated to video by an operator, the remaining 5% of false negatives or false positives could be visually corrected, bringing sensitivity and specificity to 100%.

The accuracy of pattern recognition, which is for now restricted to the most typical patterns (homogeneous, speckled, nucleolar, centromere, multiple nuclear dots and cytoplasmic) was limited.

All the manufacturers are actively continuing the development of new and more sophisticated software to offset the small defects, for a better definition in automatic recognition of patterns and light signal conversion in end-point titer. In the future, this will allow avert the need for serum dilution for titration, which will be a great advantage in economic terms and time-saving.

Table 1. Diagnosis and related number of patients studied with the automated systems for ANA-IIF screening.

Disease	No. of patients	Disease	No. of patients
SLE	21	Polymyalgia	1
Systemic sclerosis	17	Viral hepatitis (HCV)	3
Sjögren's syndrome	8	EBV infection	1
Polydermatomyositis	6	Nephrotic syndrome	1
Undifferentiated CTD	12	Rhinopathy	1
Mixed CTD	3	Chronic orticaria	1
Primary biliary cirrhosis	11	Bladder cancer	1
Fibromyalgia	1		

Table 2. IIF pattern, number, and antibody specificity (defined by methods other than IIF) of the 92 sera analyzed with the automated systems.

IIF pattern	no.	Antibody specificity	IIF pattern	no.	Antibody specificity
homogeneous	7	dsDNA	multiple nuclear dots	4	sp100
speckled	13	Ro	nuclear rim	4	gp210
speckled	5	RNP	mitotic (midbody/NuMA)	3	
speckled	4	Sm	nuclear matrix	5	hnRNP
diffuse grainy	10	Scl70	PCNA	2	cyclin
centromere	3	CENP-B	centriole	1	
nucleolar	6	PM/Scl - fibrillarin	diffuse speckles	3	DFS70
actin	1	F-actin	cytoplasmic granular	3	Jo1
mitochondrial	4	M2	ribosomial-like	3	ribP
CENP-F	1		lisosomial-like	5	
Golgi	1		vimentin-like	1	

Table 3. Positive and negative results, sensitivity and specificity of the six automated systems for ANA-IIF screening at the manufacturers' cutoff.

	Aklides	EuroPattern	Helios	Image Navigator	NovaView	G-Sight
Positive	90/92	88/92	90/92	88/92	86/92	91/92
Negative	30/34	29/34	32/34	32/34	32/34	27/34
Sensitivity %	97.8	95.7	97.8	95.7	93.5	98.9
Specificity %	88.2	85.3	94.1	94.1	94.1	79.4

Table 4. Specificity at different sensitivity values, as determined by ROC curve analysis (NA, not available).

	Sensitivity	Aklides	Europattern	Helios	Image Navigator	Nova View	G-Sight
Specificity at	**97.8%**	85.3	85.3	94.1	67.6	79.4	79.4
	95.0%	88.2	88.2	NA	91.1	82.3	85.2
	90.0%	88.2	91.1	NA	100	97.0	91.1

Table 5. ANA IIF automated/manual positive-negative agreement (PNA) per system from 12 published studies.

System	Studies (no.)	Patients (no.)	PNA (mean)
Aklides	3	1601	0.95
EuroPattern	2	565	0.96
Helios	1	1005	0.98
Image Navigator	1	3185	0.99
Nova View	2	842	0.94
Zenit G-Sight	2	562	0.92
Total	11	7760	0.98

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