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Original article

Efficiency of automated microscopy to interpret anti-nuclear antibodies in a routine laboratory by indirect immunofluorescence assay

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Abstract

Background: The detection of anti-nuclear antibody (ANA) by indirect immunofluorescence (IIF) assay is the gold standard for ANA screening in systemic autoimmune diseases. The interpretation of ANA results by human visual experts, however, is a time-consuming procedure. Recently, computer-aided automated immunofluorescence microscopy (EURO Pattern Suite) system has been used as a standard procedure in many clinical laboratories worldwide.
Objective: To compare the performance of IIF assay-based ANA detection using the automated EURO Pattern Suite with visual expert inspection.
Methods: A total of 5,000 clinical samples were collected and analyzed for ANA using the EURO Pattern Suite automated system from June to December 2021. The positive and negative results, staining patterns, and endpoint titers were compared between EURO Pattern Suite and visual readings.
Results: The sensitivity, specificity, and overall concordance of the EURO Pattern Suite were 78.4%, 97.0%, and 91.4%, respectively. The concordance between the ANA titer measurement at the same titer level generated by the automated analyzer and human specialists was 85.1% while the endpoint titers within ±1 titer difference were 96.7%. On the other hand, the concordances of pattern recognition of single and mixed patterns were 66.9% and 71.5%, respectively.
Conclusion: Despite the automated EURO Pattern Suite system's remarkably decent performance, we nonetheless recommend that the output created by the automated system be visually inspected by human specialists to ensure a high-quality standard.

Keywords*:* Anti-nuclear antibody detection, automation, indirect immunofluorescence assay, immunofluorescence microscopy, method evaluation.

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Anti-nuclear antibody (ANA) is an important indicator of autoantibodies involving in the pathology of many organ-specific systemic autoimmune diseases (SAID), including systemic lupus erythematosus (SLE), Sjogren's syndrome, mixed connective tissue disease (MCTD), systemic sclerosis (SSC), and rheumatoid arthritis (RA).[1-7] At present, many ANA determination systems are accessible for clinical laboratory diagnosis. Each of them uses different antigen sources and detection systems.[8-11] Indirect immunofluorescence assay using HEp-2 cells (a cancerous human epithelial cell line) has been used as a gold standard test recommended by the American College of Rheumatology (ACR) for ANA screening because this method can assess 100 - 150 cell antigens at different stages of the cell.[1]

Additionally, using the HEp-2 cell-based IIF assay is exceptionally informative because it can reveal the levels and the patterns of ANA related to clinical outcomes. In general, HEp-2 cell-based IIF assay is visualized and interpreted by clinical laboratory experts conventionally. This process is disadvantageous and time-consuming because it requires experienced clinical laboratory technicians and is prone to the inconsistency of intra-and inter-laboratory interpretation. Recently, a consensus nomenclature of ANA IIF staining patterns has been created as the International Consensus on ANA Patterns (ICAP) that identifies the clinical relevance of the 29 distinct Hep-2 IIF patterns. Specifically, each pattern is characterized by alphanumeric AC codes (AC 0 - 29) and associated with the description of target antigens, disease associations, and references.[1-3,12-15] Hereafter, numerous commercial automated platforms equipped with software-based image acquisition, analysis, and evaluation systems for ANA IIF assay have been developed to mitigate the disadvantage posed by the conventional expert-based interpretation.[16,17] Despite this, concerns about the accuracy of the automated systems in the interpretation of the ANA IIF assays have been raised. To date, there are at least six commercially available automated platforms systems.[6,18] The EURO Pattern Suite is one of the most customary automated systems for analyzing ANA IIF assays widely used in clinical laboratories in many countries.[1,4,12,19-21] We intended to determine a proficient and expeditious workflow for ANA testing by integrating the EURO Pattern Suite in our routine laboratory system. In this study, we assessed the performance of the computer-aided immunofluorescence microscopy system, EURO Pattern Suite, by comparing its interpretation accuracy with the results given by clinical laboratory experts.

Methods and methods

Serum samples

A total of 5,000 serum samples were collected from the samples submitted for routine IIF-based ANA testing by various clinical departments at King Chulalongkorn Memorial Hospital from January 2018 to October 2019. The informed consent and patient anonymity were preserved using methods approved by the Internal Review Board of the Faculty of Medicine, Chulalongkorn University (IRB no. 559/64). The flow diagram of patient sample selection is depicted in Figure 1.

Automated indirect immunofluorescence assay

ANA tests were processed by the IF-Sprinter (Euroimmun, Lübeck, Germany) using the ANA Mosaic 1A EURO Pattern standard kit (Euroimmun, Lübeck, Germany). Each slide contained ten reaction fields in which each area consisted of two BIOCHIPs (HEp-20-10 and monkey liver cells). Serum samples were diluted in PBS-Tween buffer including the fluorescein isothiocyanate (FITC)-labeled anti-human antibodies (goat) conjugated with propidium iodide. After image acquisition, cell images were segmented and analyzed by EURO Pattern Suite software. The evaluation and interpretation of the assays were performed according to the manufacturer's instructions. As for ANA titer measurement, we prepared sera by performing two-fold serial dilution starting from 1:80 to 1:640 dilution ratio. The interpretation was classified according to the manufacturer's instructions as a negative result when the fluorescence intensity was less than that of the 1:80 dilution; a positive result when the fluorescence intensity was equal to or greater than the 1:80 dilution; a borderline result when the fluorescence intensity was close to cut-off and manual interpretation by human experts was required for positive/negative determination; and no interpretation when the software could not provide a conclusive result and manual interpretation by human experts was required.

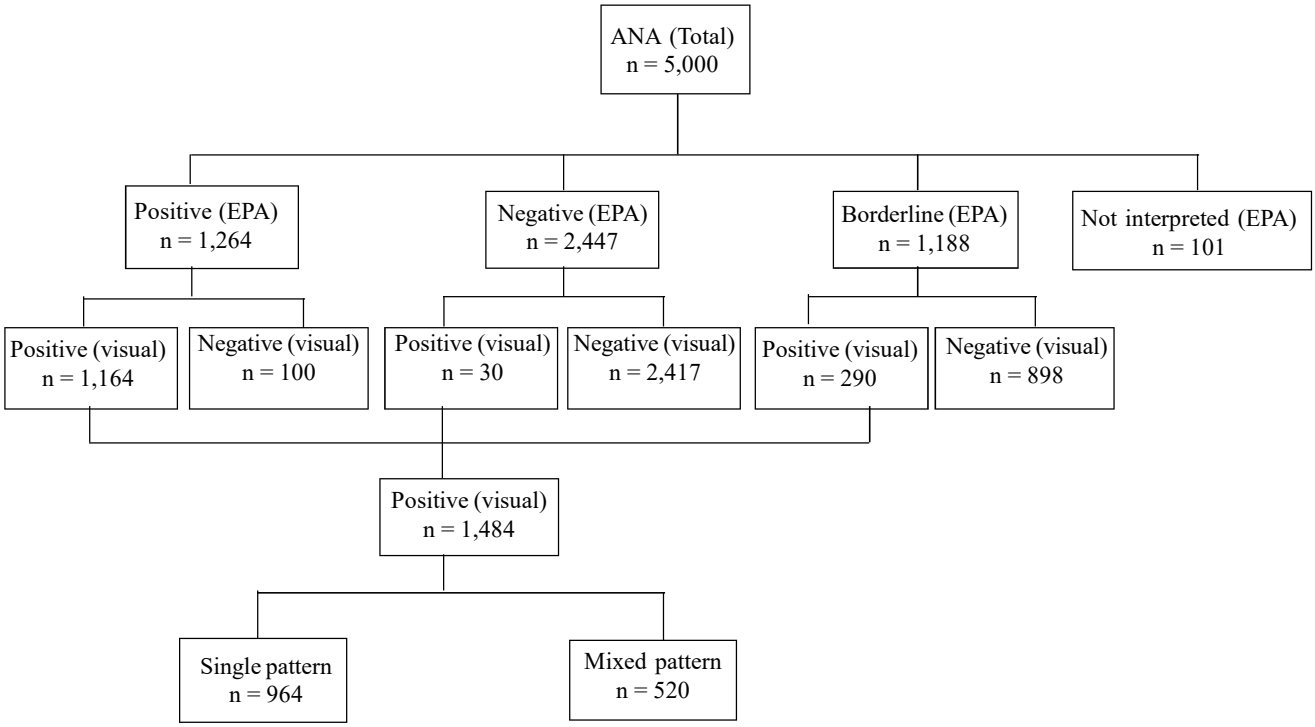

Figure 1. Flowchart for sample evaluation using EURO Pattern Suite automated immunofluorescence microscopy system. EPA: EURO Pattern Suite software; visual: visual inspection by human specialists; ANA: anti-nuclear antibody;N: number of samples; Positive: ANA positive; Negative: ANA negative; Borderline: neither positive or negative (with titer and patterns reported); Not interpreted: unclassified (without titer and patterns reported).

After slide preparation, slides were placed on a computer-aided EURO Pattern Suite microscope, and cell images were recorded automatically. Based on the reference image database provided by the Euroimmun company, the samples were then evaluated and assigned their classification result. For positive ANA samples, their cell images were interpreted by the software in multiple steps as follows: 1) classification of images as negative/positive/ borderline; 2) image pattern recognition (homogeneous, speckled, nucleolar, centromere, nuclear dot, nuclear membrane, cytoplasmic); and 3) titer determination of each pattern (< 1:80, 1:80, 1:160, 1:320, 1:640, 1:1,280, and ≥ 1:2,560).[22] In parallel, the images were also evaluated by at least two clinical laboratory specialists.

Statistical analysis

Correlation between the results interpreted by the EURO Pattern Suite software and visual experts was determined using the percentage of concordance and the Cohan's kappa coefficient (*K*), *K* - value calculation was performed in Microsoft Excel (version 16.71) and calculated using the following equation:

$$k = \frac{f_0 - f_e}{N - f_e}$$

where f_0 is hypothetical frequency, f_e is observed frequency, and N is number of samples. $K < 0$ indicates no agreement, 0 - 0.2 indicates little agreement, 0.21 - 0.40 indicates small agreement, 0.41 - 0.60 indicates moderate agreement, 0.61 - 0.80 indicates good agreement, and 0.81 - 1.0 indicates almost perfect agreement.[1, 12] Similarly, the performance of the software was further evaluated for its sensitivity and specificity by comparing the results determined by the software and the reference results determined by visual experts.

Results

Prediction of ANA samples

Based on 5,000 patient samples submitted for automated routine ANA testing, 101 samples could not be interpreted by EURO Pattern Suite software. As such, the remaining 4,899 samples were used in further analysis. Of these samples, 1,164 samples were identified by both EURO Pattern Suite software and visual experts as ANA positive and 3,315 samples as ANA negative. In contrast, the software incorrectly identified 100 samples as false positive and 320 samples as false negative (Table 1). The percentage of concordance (91.4%, K = 0.79) and specificity (97.1%) were relatively high, indicating that the performance of ANA classification done by automated software was equivalent to evaluation determined by the clinical laboratory experts. On the other hand, the sensitivity of the software was moderately acceptable (78.4%). As for the samples incorrectly identified by the EURO Pattern Suite software as false positive, the titers (1:160, 1:80 and 1:320) demonstrated a high percentage of error and the patterns associated with the high error rate included speckled type and homogeneous type (Table 2).

Measurement of ANA titers

In ANA testing with a series of serum dilutions, we compared the endpoint titers determined by visual experts and the software (Table 3). We found that 4,168 samples were determined to have the same titer level by visual experts and the software, resulting in 85.1% (K = 0.68) of concordance. In contrast, considering the samples with ± 1 titer difference (n = 4,735), the concordance was improved to 96.7% (K = 0.93).

Classification of ANA patterns

To determine the performance of the software in ANA pattern interpretation, we divided ANA positive samples into two groups (single and mixed patterns) and evaluated the results interpreted by the software comparing to the clinical laboratory experts. The results showed that single patterns, including homogeneous, centromere, nuclear dot, nuclear membrane and cytoplasmic, in ANA positive samples determined by the software were consistent with the results determined by the clinical laboratory experts (Table 4). However, when including other samples with mixed patterns, missing patterns, and ANA negative as determined by the software, the pattern classification performance remained at a high level for some patterns, such as cytoplasmic, nuclear dot, homogeneous type, and nuclear membrane patterns (88.5%, 87.5%, 81.1%, and 80.0%, respectively). In particular, while the software could correctly identify centromere patterns at an acceptable rate (74.2%), it failed detrimentally to determine the patterns such as speckled type and nucleolar type (42.6% and 42.6%, respectively). Taken our results together, the overall performance of the software in pattern classification of positive ANA samples was 66.9% (K = 0.58) compared to the clinical laboratory experts.

Moreover, in ANA positive samples, especially with mixed patterns (defined as the samples with two or more nuclear patterns regardless of cytoplasmic pattern), our analysis revealed that the overall performance of the software to recognize these patterns was still at an acceptable level (71.5%, K = 0.6) (Table 5). However, the percentage of concordance varies widely from one major pattern to another found in mixed pattern samples, such as homogeneous/speckled (83.6%), nuclear membrane (68.2%), nucleolar (59.1%), centromere (57.1%), and nuclear dot (52.8%) pattern.

Table 1. Comparison between interpretation by visual experts and EURO Pattern Suite software.

	Visual evaluation (n = 4,899)	
	Positive	**Negative**
EURO Pattern		
Positive	1,164	100
Negative	320	3,315
Sensitivity	78.4%	
Specificity	97.1%	
Concordance	91.4%	
K - value (agreement)	0.8	

Table 2. ANA patterns and titers of false positive by EURO Pattern Suite software (n = 100).

Pattern / Titer	HT	ST	NT	NM	ND	CEN	> 1 patterns	% error
1:80	9	11	2	2	-	-	1	25
1:160	13	15	-	1	7	-	7	43
1:320	-	1	-	12	3	-	4	20
1:640	2	-	1	3	-	-	1	7
1:1,280	-	1	-	-	-	-	2	3
1:2,560	-	-	-	-	1	-	-	1
≥ 1:2,560	-	-	-	-	-	1	-	1
% error	24	28	3	18	11	1	15	-

HT: homogeneous type, ST: speckled type, NT: nucleolar type, NM: nuclear membrane,
ND: nuclear dot, CEN: centromere.
The percent error is calculated by dividing the number of false positive samples in a particular pattern by the total number of false positive samples across all patterns and multiplying the result by 100.

Table 3. Correlation of titer level determined by visual experts and EURO Pattern Suite software.

EURO Pattern Suite	Visual evaluation (authorized)							
Titer	< 1:80	1:80	1:160	1:320	1:640	1:1,280	1:2,560	≥ 1:2,560
< 1:80	3,315	309	8	3	0	0	0	0
1:80	25	35	2	0	0	0	0	0
1:160	43	83	197	2	0	0	0	0
1:320	20	23	29	242	16	0	0	5
1:640	7	3	15	62	105	2	0	3
1:1,280	3	1	1	3	16	37	0	10
1:2,560	1	0	0	2	6	1	38	19
≥ 1:2,560	1	1	1	1	2	1	1	199
Total	3,415	455	253	315	145	41	39	236

Table 4. Correlation of the ANA patterns from single pattern group classified by visual experts and EURO Pattern Suite software.

Single pattern	Visual evaluation (authorized)								Total
	HT	ST	NT	CEN	ND	NM	PCNA	Cyto	
EURO Pattern Suite									
Homogeneous type	116	73	2						191
Speckled type		209				1	1		211
Nucleolar type			40						40
Centromere		1		23					24
Nuclear dot		5			7				12
Nuclear membrane		9				12			21
PCNA (not evaluated)									0
Cytoplasmic pattern								582	582
Extra pattern	12	98	34	30	6				180
Missing pattern	27	194	52	8	1	2	1	73	358
Total	155	589	128	61	14	15	2	655	1,619
% accuracy	81.1	42.6	42.6	74.2	87.5	80	0	88.9	
K - value	0.78	0.33	0.41	0.74	0.87	0.80	0	0.81	
Total accuracy					66.9%				
K - value					0.58				

PCNA: proliferating cell nuclear antigen, Extra pattern: EPA software could be evaluated ANA patterns more than visualization experts, Missing pattern: EPA software could not be evaluated ANA patterns.

Table 5. Correlation of the ANA patterns from mixed pattern group classified by visual experts and EURO Pattern Suite software.

	Visual evaluation (authorized)					
Mixed pattern	**HT/ST**	**NT and other**	**CEN and other**	**ND and other**	**NM and other**	**Total**
EURO Pattern Suite						
HT/ST	244	4	1	3	2	254
NT and other	5	88	2	1	-	96
CEN and other	-	-	12	-	-	12
ND and other	3	2	3	19	1	28
NM and other	6	1	-	2	15	24
missing pattern	34	54	3	11	4	106
Total	292	149	21	36	22	520
% accuracy	83.6%	59.1%	57.1%	52.8%	68.9%	
K - value	0.68	0.50	0.56	0.50	0.67	
Total accuracy			71.5%			
K - value			0.59			

HT: homogeneous type, ST: speckled type, NT: nucleolar type, CEN: centromere, ND: nuclear dot, NM: nuclear membrane, missing pattern: EPA software could not be evaluated ANA patterns

Discussion

In this study, we used HEp-2 cell-based indirect immunofluorescence assay as a gold standard for ANA testing, and we analyzed ANA patterns of 5,000 samples by visual experts and computer-aided analysis by EURO Pattern Suite software, and evaluated the performance of the software by comparing it to the results given by the visual experts. Our analyses showed that the classification of ANA samples made by the software was in high agreement with the decision made by the visual experts. However, the performance of the software still has a shortfall in sensitivity as it made 100 false positives and 320 false negative predictions. The overall performance of the software in the determination of the endpoint titer of ANA samples could achieve an acceptable level. Although the software could accomplish a moderate accuracy level in classifying ANA patterns, including single and mixed pattern populations, its performance was better in pattern classification of single-pattern ANA samples than mixed pattern samples. In particular, it failed to identify rare ANA patterns such as proliferating cell nuclear antigen (PCNA) and some groups of component-level patterns, including dense fine speckled (DFS), anti-mitochondrial (AMA)-like pattern, Golgi apparatus, and rods and rings patterns, similar to the previous reports.[13, 14]

Therefore, the limitations of our study are strictly limited to A) the ability of the EUROP attern Suite software to recognize most single patterns (homogeneous, centromere, nuclear dots, nuclear membrane and cytoplasmic); B) a restriction of the EUROPattern Suite that requires the use of Euroimmun IIF to ensure high quality of results, in which the substrate and the biochips require constant quality control check; and C) a multi-center study is required to rule out the possibility of interobserver reading bias.

Despite these drawbacks, computer-aided immunofluorescence microscopy systems such as EURO Pattern Suite can help eliminate human errors in sample preparation and interpretation. Not only the automated system can execute a high-throughput analysis, but it can also handle laboratory management tasks simultaneously, including registration and transfer of patient data, with precision and ease. From our evaluation, when it came to ANA prediction and ANA titer determination, the automated system achieved a decent performance. However, ANA pattern outputs needed to be inspected visually by human specialists to ensure the high-quality standard. Furthermore, as image analysis techniques and software for ANA testing are developed, the classification performance of this automated microscopy system can be greatly improved, allowing for faster high-throughput automated clinical diagnosis.

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Conflicts of interest statement

Each of the authors has completed an ICMJE disclosure form. None of the authors declare any potential or actual relationship, activity, or interest related to the content of this article.

Data sharing statement

The present review is based on the references cited. Further details, opinions, and interpretation are available from the corresponding authors on reasonable requests.

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