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DIAGNOSTICS AND ENVIRONMENTAL FACTORS

A fully automated IIF system for the detection of antinuclear antibodies and antineutrophil cytoplasmic antibodies

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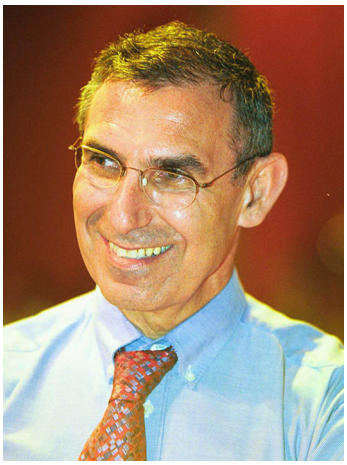

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Abstract Indirect immunofluorescence (IIF) is the main technique for the detection of antinuclear antibodies (ANA) and antineutrophil cytoplasmic antibodies (ANCA). The fully automated IIF processor HELIOS® is the first IIF processor that is able to automatically prepare slides and perform automatic reading. The objective of the present study was to determine the diagnostic performance of this system for ANA and ANCA IIF interpretation, in comparison with visual IIF. ANA detection by visual IIF or HELIOS® was performed on 425 sera samples including: 218 consecutive samples submitted to a reference laboratory for routine ANA testing, 137 samples from healthy subjects and 70 ANA/ENA positive samples. For ANCA determination, 170 sera samples were collected: 40 samples for routine testing, 90 samples from healthy blood donors and 40 anti-PR3/anti-MPO positive subjects. Good correlation was found for the visual and automated ANA IIF approach regarding positive/negative discrimination of these samples (kappa = 0.633 for ANA positive samples and kappa = 0.657 for ANA negative samples, respectively). Positive/negative IIF ANCA discrimination by HELIOS® and visual IIF revealed a complete agreement of 100 % in sera from healthy patients and PR3/MPO positive samples (kappa = 1.00). There was 95 % agreement between the ANCA IIF performed by automated and visual IIF on the investigation of routine samples. Based on these results, HELIOS® demonstrated a high diagnostic performance for the automated ANA and ANCA IIF interpretation that was similar to a visual reading in all groups of samples.

Keywords Antinuclear antibodies · Antineutrophil cytoplasmic antibodies · Indirect immunofluorescence · Automation · Autoantibodies · Autoimmunity

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Abbreviations

ANA	Antinuclear antibodies
ENA	Extractable nuclear antigens
ANCA	Antineutrophil cytoplasmic antibodies
cANCA	Cytoplasmic ANCA
pANCA	Perinuclear ANCA
MPO	Myeloperoxidase
PR3	Proteinase 3
ELISA	Enzyme-linked immunosorbent assays
IIF	Indirect immunofluorescence
SLE	Systemic lupus erythematosus
RA	Rheumatoid arthritis
SSc	Systemic sclerosis
IIM	Idiopathic inflammatory myopathies
SjS	Sjögren's syndrome
HEp-2	Human epidermoid laryngeal carcinoma

ACR American College of Rheumatology
AAV ANCA-associated vasculitis

Introduction

The detection and measurement of autoantibodies against nuclear and cytoplasmic antigens is an essential step in the serological diagnosis of systemic autoimmune diseases. In some cases, their presence may assist in the prognosis, the subclassification and the monitoring of disease activity [1, 2]. Given this central role, antinuclear antibodies (ANA) and antineutrophil cytoplasmic antibodies (ANCA) screening must be highly specific, accurate and reproducible.

For several decades, visual indirect immunofluorescence (IIF) on HEp-2 (human epidermoid laryngeal carcinoma) cells has been the reference technique for the first step of ANA testing for the diagnosis of systemic autoimmune rheumatic diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), systemic sclerosis (SSc), idiopathic inflammatory myopathies (IIM) and Sjögren's syndrome (SjS) [3]. Conversely, the IIF method is burdened by some undesirable factors including the need for expert morphologists, subjectivity of interpretation, intra- and inter-laboratory variability, and low standardization and automation. These drawbacks increase the cost and the time required for ANA and ANCA screening by IIF. Therefore, this method has been challenged by novel techniques based on solid-phase immunoassays (e.g., ELISA, dot/line immunoassay and addressable bead/microarray assays) [4–7]. These methods can be automated and are more cost efficient particularly in light of the rising diagnostic demand that stems from the growing clinical impact of autoimmune diseases. However, high rates of false-negative findings have been reported for these techniques [4, 8]. Addressing this issue, the respective American College of Rheumatology (ACR) task force confirmed IIF as the gold standard for ANA testing [4].

Screening for ANCA plays a key role in the serological diagnosis of ANCA-associated vasculitis (AAV) [9]. According to the recommendations for ANCA diagnostics, positive findings of standard screening tests by IIF on ethanol-fixed neutrophils must be confirmed by ELISAs [10]. Depending on ethanol-fixed neutrophils IIF pattern, ANCA can be subclassified into cytoplasmic ANCA (cANCA) and perinuclear ANCA (pANCA) patterns. Due to the fact that anti-MPO ANCA and antinuclear antibodies (ANAs) may demonstrate similar IIF patterns on ethanol-fixed neutrophils, IIF on formalin-fixed neutrophils is used for their discrimination [11]. In contrast to ANA detection on HEp-2 cells, polymorphonuclear granulocytes are characterized by varying shapes of the nucleus, which is usually lobed into three segments. Therefore, IIF identification of granulocyte staining patterns is more complicated and is also associated with all the main drawbacks of this method. Nevertheless, most of the commercially available ELISAs for ANCA detection have been reported to be inferior to IIF in terms of sensitivity [12].

In order to automate and standardize ANA and ANCA screening by IIF, different commercially available platforms were developed [13–25]. These systems are based on the automation of all of the steps of IIF procedure, including the preparation of substrates and the microscope reading.

Some of these systems can only distinguish between positive and negative screening results (HELIOS®, Aesku.Diagnostics, Wendelsheim, Germany; Image Navigator, Immuno Concepts, Sacramento, USA; Cytospot, Autoimmun Diagnostika, Straßberg, Germany), whereas others are also able to classify basic staining patterns (AKLIDES, Medipan, Dahlewitz/Berlin, Germany; Nova View, Inova, San Diego, USA; Zenit G Sight, A. Menarini Diagnostics, Grassina-Firenze, Italy; EUROPattern, Euroimmun, Lübeck, Germany) [21–23]. The comparison of the diagnostic accuracy of six systems for automated ANA IIF reading and visual IIF performed on the same sera revealed an overall sensitivity and specificity of 96.7 and 89.2 %, respectively [23].

The objective of the present study was to determine the diagnostic performance of the fully automated (all in one box) IIF processor HELIOS® for automatic slides reading of ANA and ANCA samples, specifically in discrimination between positive and negative samples in comparison with visual IIF.

Materials and methods

Sera samples

ANA detection by visual IIF or HELIOS® was performed on 425 sera samples including: 218 consecutive samples submitted to a reference laboratory for routine ANA testing, 137 samples from healthy subjects and 70 ANA/ENA positive samples.

For ANCA determination, 170 sera samples were collected: 40 samples for routine testing, 90 samples from healthy blood donors and 40 anti-PR3/anti-MPO positive subjects. The preliminary selection for ANA and ANCA positivity was made in our laboratory.

The study fulfilled the ethical guidelines of the most recent declaration of Helsinki and received approval by the local ethical committees (Edinburgh, 2000).

Characteristics of HELIOS® [25]

This system is a new platform capable of performing all immunofluorescence steps automatically and continuously from start to finish without human intervention.

Briefly, this platform consists of two built-in barcode readers for samples and slides and an auto-focus epifluorescence microscope unit. The sample barcode reader ensures sample traceability and eliminates time and error spent entering long patient ID numbers. Slide barcode reader ensures slide traceability. The *AESKUSLIDES*® IFA reagents are barcoded with all necessary information (reference, lot, expiry date, etc.) including a unique serial number. This unique feature delivers front-end traceability for the whole process ensuring compliance with laboratory accreditation. The integrated microscope (incorporating Nikon-based optics) is complemented by the AESKU engineered motor ensuring both accuracy and speed.

The HELIOS® software processing analysis is done by a mathematical algorithm which analyzes every IIF pattern and suggests a pre-classification result (positive/negative). This system uses the HELMED® IFA v3.0 Software which is a well established and robust version. All *AESKUSLIDES*® IFA kit protocols are validated and provided.

The image capturing also works automatically. It can be used independently from other modules or can start automatically after the slide processing is completed.

The user-predefined number of pictures per well (between 1 and 10) is generated.

For final classification, all informations (patient ID, lot numbers, etc.) including pictures of the wells are presented in this simplified interface. The pre-classification between positive and negative samples can be selected to save time and focus mainly on positive and/or borderline results. A broad spectrum of patterns can be correctly recognized by HELIOS® as positive, including homogeneous, speckled, nucleolar, nucleolar dots, centromere, multiple nucleolar dots, cytoplasmic and cytoskeleton.

After confirmation of the results, a pattern can be assigned manually by the operator and a follow-up decision can be made, ensuring secure management of results.

Visual interpretation of HEp-2 assay

Detection of ANA was performed by a commercial ANA IIF assay using HEp-2 cells (Bio-Rad Laboratories, CA, USA) described elsewhere [13]. The processed slides were read visually by two operators using a fluorescent microscope.

Visual interpretation of ANCA assay

Determination of ANCA was done by ANCA IIF assay (Inova Diagnostics, San Diego, CA, USA).

Statistics

The degree of agreement between the visual and the automated antibody pattern interpretation was assessed by the percentage of concordance and by kappa coefficients. According to Altman [26], kappa (k) values were interpreted as follows: ≤0.20 poor, 0.21–0.40 fair, 0.41–0.60 moderate, 0.61–0.80 good and 0.81–1.00 very good agreement.

Table 1 ANA IIF testing: comparison between the interpretations of two observers on total samples ($n = 425$)

ANA findings	Kappa coefficient	Agreement consideration
Positive	0.639	"Good"
Negative	0.629	"Good"

Table 2 ANA IIF testing: comparison between the interpretations of an observer and HELIOS® on total samples ($n = 425$)

ANA findings	Kappa coefficient	Agreement consideration
Positive	0.633	"Good"
Negative	0.657	"Good"

Table 3 ANCA IIF testing: comparison between the interpretations of an observer and HELIOS® on total samples ($n = 150$)

Groups of samples	HELIOS®	Visual IIF	Agreement (%)
Routine sera (No = 40)	All negative	All negative	100
Healthy subjects (No = 90)	All negative	All negative	100
Anti-PR3/anti-MPO samples that were previously identified as positive (No = 40)	38 positive	40 positive	95

Results

Referring to the total of 425 samples, there was a good agreement between two observers in visual ANA IIF interpretation (kappa = 0.639 for ANA positive samples and kappa = 0.629 for ANA negative samples, respectively) (Table 1). Similarly, a good correlation was found for the visual and automated ANA IIF approach regarding positive/negative discrimination of these samples (kappa = 0.633 for ANA positive samples and kappa = 0.657 for ANA negative samples, respectively) (Table 2).

The positive/negative IIF ANCA discrimination by HELIOS® and visual IIF revealed a complete agreement of 100 % in sera from healthy patients and PR3/MPO positive samples (kappa = 1.00). There was 95 % agreement between the ANCA IIF performed by automated and visual IIF on the investigation of routine samples (Table 3).

Discussion

The automation of IIF processing is a major step toward the standardization of this method. Standardization is a crucial concern because of intra- and inter-laboratory variations, subjective image interpretation, individual experience and capability of the laboratory staff. Systems for automated IIF evaluation may contribute to the reduction of errors and improve the standardization of ANA and ANCA IIF testing [3].

The objective of this study was to compare the results of ANA and ANCA recognition by visual subjective IIF reading and IIF obtained by the HELIOS® IIF processor.

The basic requirement for the use of automated interpretation systems in routine diagnostics is the correct and reproducible differentiation of positive and negative samples.

Since the detection of ANA and ANCA is employed as serological screening for patients with suspected rheumatic disorders on the one hand and is part of the classification criteria of systemic rheumatic diseases on the other hand, three different groups of sera were tested. There were sera submitted to our laboratory for routine ANA and ANCA testing, samples from healthy subjects and ANA/ENA and anti-PR3/MPO positive samples.

As shown in the results, a good correlation was found between visual ANA IIF interpretations performed by two different observers (Table 1). Similarly, there was a good agreement for the classification of positive and negative ANA samples between automated and visual assessment by experienced examiners in different patient cohorts (kappa = 0.633 for ANA positive samples and kappa = 0.657 for ANA negative samples, respectively) (Table 2).

Moreover, positive/negative IIF ANCA discrimination by automated and visual approach revealed a complete agreement of 100 % in sera from healthy subjects and PR3/MPO positive samples, and a very good agreement of 95 % on the investigation of routine samples (Table 3).

Based on these results, HELIOS® demonstrated a high diagnostic performance for the discrimination between ANA and ANCA positive and negative samples and revealed no difference to visual reading in all groups of samples. Although this IIF processor does not provide an automated interpretation of the ANA pattern, it can correctly recognize many patterns as positive including homogeneous, speckled, nucleolar, nucleolar dots, centromere, multiple nucleolar dots, cytoplasmic and cytoskeleton. A specific pattern can later be assigned by an operator using a large pattern library.

Recently, several studies have established the efficiency of different automated IIF systems for objective ANA detection providing the basis for the employment of IIF as gold standard for ANA testing according to recommendations of ACR [13–24]. Advanced stages of research were performed on six commercial systems: EUROPattern, AKLIDES, Nova View, HELIOS®, Zenit G Sight and Image Navigator.

By comparing these automated systems for ANA IIF interpretation on the same series of sera, it has been recently shown that all of them are capable for screening with a total sensitivity rate of 96.7 % and specificity rate of 89.9 % [23].

Some of these systems, including AKLIDES, Nova View, Zenit G Sight and EUROPattern, are capable of providing ANA pattern recognition of fluorescence images. Homogeneous, speckled, nucleolar, centromeric and cytoplasmic patterns may be recognized by all four systems listed above, while three of them (EUROPattern, AKLIDES and Nova View) are able to identify the nuclear dot pattern. However, it has been found that these automated systems have a limitation in the identification of some patterns. In this manner, EUROPattern managed to identify correctly ANA patterns in 79 % of the cases, AKLIDES in 52 %, Nova View in 54 % and Zenit G Sight in 63 %. While the classic nuclear (homogeneous, speckled and centromeric) and nucleolar patterns are identified in 70–85 % of the cases, the rarer patterns (multiple nuclear dots, nuclear rim, midbody, PCNA and nuclear matrix) are found at a significantly lower rate of 25–50 % [23].

Several factors may impact the correct negative/positive classification of samples by automated systems, including the pattern of immunofluorescence and antibodies' titers. It should be noted that mixed patterns influence IIF ANA detection by automated systems, because dominant autoantibodies (or unspecific antibodies) may complicate pattern differentiation. As reported for the AKLIDES [13] and EUROPattern [16], distinction of patterns with two or more autoantibodies can be difficult, depending on their number, target and titer. Titering of the samples (at least two dilutions) is recommended to facilitate the interpretation of mixed patterns.

Besides ANA screening, accumulated data including the results reported in this study have shown a high agreement between visual and automated interpretation of IIF testing for ANCA assessment [17, 19, 20, 24].

While the HELIOS® has all the advantages of novel automated IIF screening for serology of systemic autoimmune rheumatic diseases, it also includes several unique features [25]. As listed above, this is the first system that is able to automatically prepare slides. This ability, along with automatic reading, contributes to the homogenous working protocol and allows for walkaway processing and high throughput. Due to the growing demand for ANA detection, the efficiency of this technique is especially

important in clinical practice and represents a great advantage in economic terms and time saving.

As well as other automated systems used in IIF processing, the HELIOS® provides standardization of the IIF process by automatic reading of the light signal and expressing quantitative results on a continuous scale which may reduce the subjectivity and intra- and inter-laboratory variations associated with visual IIF reading.

All automated systems include a short final step of approving positive results, in which the investigator can confirm, modify the results (if necessary) or decide to send the samples to a second-level test that is able to detect the presence of several antibodies. This is especially important in differentiating of mixed patterns and in evaluating of weakly positive sera. All of the automated systems have a high sensitivity rate, but the cutoff may also be modified by investigator according to the clinical requirements.

Generally, large laboratories that deal with a large number of samples perform a two-step IIF diagnostic process. The first step includes a positive/negative screening that is done with a particular screening dilution (e.g., 1:80 or 1:100), and the second step consists of additional dilution carried out for positive samples. In this regard, HELIOS® is able to combine the results of all available dilutions into one final result per patient and display it along with the IIF images on a single patient-specific report form. It should be noted that the verification of negatives significantly shortens the analysis procedure and assists in focusing attention on positive results.

In conclusion, the current study observed a good agreement between visual and automated ANA and ANCA IIF interpretation and proved a high diagnostic performance of HELIOS®. In addition to automated IIF interpretation, this system automatically prepares slides that may significantly increase the laboratory efficiency and contribute to the standardization of the IIF process. Further studies are warranted to evaluate this fully automated novel system for ANA and ANCA IIF screening in clinical immunologic laboratories.

Conflict of interest The authors have no conflicts of interest to declare.

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