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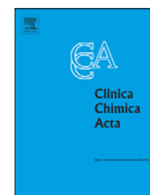

Detection of antinuclear antibodies by automated indirect immunofluorescence analysis

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ABSTRACT

Background: Testing for antinuclear antibodies is useful for the diagnosis of systemic rheumatic diseases. Automated systems for image acquisition and interpretation of indirect immunofluorescence-based tests are increasingly used. The diagnostic performance of such automated approach in untreated patients has not been reported.

Methods: Antinuclear antibodies were measured by automated indirect immunofluorescence using Zenit G. Sight on HEp2 and HEp2000 substrate in 268 consecutive samples submitted to the laboratory for antinuclear antibody testing, and in 231 patients with a systemic rheumatic disease at the time of diagnosis, 143 blood donors, 134 patients with chronic fatigue syndrome, and 133 diseased controls.

Results: Image acquisition by G-Sight was of high quality. The accuracy of pattern assignment was limited. There was a significant correlation between automated estimation of fluorescence intensity (probability index of positivity) and end-point titer. Probability index interval specific likelihood ratios for systemic rheumatic disease increased with increasing level of positivity probability. With the HEp-2 substrate, the likelihood ratio for systemic lupus erythematosus was 0.06, 0.4, 6.8, 12.1, and 43.9 for a probability measure of positivity of ≤10, 11–≤30, 31–≤50, 51–≤85, and >85, respectively.

Conclusion: Quantitative data generated by automated image acquisition facilitates standardized interpretation.

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1. Introduction

Antinuclear and anti-cytoplasmic antibodies are important diagnostic markers for systemic rheumatic diseases and autoimmune hepatitis [1–3]. Traditionally, indirect immunofluorescence on Hep-2 cells is used to screen for antinuclear antibodies. More specific, second line tests are performed to identify the target antigen of the antibodies (e.g. dsDNA or extractable nuclear antigens). Although quantitative (multiplexed) solid phase immunoassays for specific antibodies can be automated, they cannot fully replace indirect immunofluorescence for antinuclear antibody testing, because of the lower sensitivity [4,5]. Therefore, recent recommendations state that immunofluorescence antinuclear antibody testing should remain the gold standard [6].

Indirect immunofluorescence, however, suffers from low-throughput and intra- and inter-laboratory variance. Visual evaluation is time consuming, subjective and requires considerable expertise of the technicians [7]. To overcome these shortcomings, automated approaches for indirect immunofluorescence analysis are being developed [8]. The Aklides system (Medipan, Germany) was the first automated system for indirect immunofluorescence analysis of antinuclear antibodies. Good agreement between Aklides reading and visual reading has been reported [7,9,10]. Other systems are currently being developed and/or introduced in autoimmune laboratories. Few studies are available on the performance of automated indirect immunofluorescence analysis and none of these studies have assessed the clinical performance characteristics of such systems in patients who are presenting for diagnosis of a systemic rheumatic disorder.

In the present study we evaluated detection of antinuclear antibodies by G-Sight (Menarini), an automated system for image acquisition and interpretation of indirect immunofluorescence-based tests. We evaluated the ability of the system (i) to estimate the fluorescence intensity and (ii) to correctly classify fluorescence

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patterns. A major objective of the study was to determine the diagnostic performance of G-Sight for systemic lupus erythematosus and other connective tissue diseases in a cohort of untreated patients presenting for diagnosis and controls.

2. Materials and methods

2.1. Study population

A first cohort consisted of samples with mono-specific antibodies, including antibodies [i] to centriole (n = 3), centromere (n = 37), the cytoplasm (fibrillar pattern) (n = 7), nuclear membrane (n = 14), midbody (n = 8), proliferating cell nuclear antigen (n = 8), mitotic spindle (n = 16) [detected by indirect immunofluorescence], and [ii] Scl-70 (n = 39), RNP (n = 26), and SSA (n = 48) [detected by EliA (Thermo-Fisher).

A second cohort consisted of 268 consecutive samples submitted to the laboratory for analysis of antinuclear antibodies (male/female ratio: 90/178, mean age 49 years, range 4–84 years).

A third cohort consisted of well-characterized patients with a systemic rheumatic disease. This included patients with systemic lupus erythematosus (n = 77, male/female ratio 10:67, mean age 37 years, range 15–72 years), cutaneous lupus (n = 10, male/female ratio 3:7, mean age 49 years, range 32–85 years), systemic sclerosis (n = 69, male/female ratio 25:44, mean age 53 years, range 18–79 years), mixed connective tissue disease (n = 13, male/female ratio 1:12, mean age 31 years, range 16–66 years), primary Sjögren's syndrome (n = 35, male/female ratio 11:17, mean age 50 years, range 21–75 years), polymyositis/dermatomyositis (n = 27, male/female ratio 11:17, mean age 54 years, range 26–77 years). All samples included in this study were obtained from patients visiting a systemic disease clinic, and had a diagnosis assigned as a result of that visit. None of the patients had received any immunosuppressive medication.

The control groups included: healthy blood donors (n = 143, male/female ratio 70:73, mean age 44 years, range 19–65 years), patients with chronic fatigue syndrome (n = 134, male/female ratio 24:110 mean age 41 years, range 18–72 years), and diseased controls (n = 133, male/female ratio 33:100, mean age 46 years, range 17–81 years). The diseased controls were consecutive patients who consulted the rheumatology clinic and for whom the rheumatologist considered it important to request antinuclear antibodies. Afterwards, a diagnosis of systemic rheumatic disease was excluded (rheumatoid arthritis was also excluded). Disease characteristics of primary Sjögren's syndrome conformed with the American-European consensus classification criteria [11]. Patients with systemic lupus erythematosus and scleroderma met the classification criteria of the American College of Rheumatology [12,13]. Patients with polymyositis/dermatomyositis met the criteria of Bohan and Peter [14], and patients with mixed connective tissue disease met the criteria of Alarcon-Segovia [15].

All serum samples were obtained as part of routine screening for autoantibodies. No informed consent was needed for this retrospective study, but the study was approved by the local ethics committee.

2.2. Detection of antinuclear antibodies

Antinuclear antibodies were detected by Zenit G-Sight (A. Menarini Diagnostics, Florence, Italy), an automated system for image acquisition and interpretation of indirect immunofluorescence-based tests. The automated fluorescence microscope uses a LED light source (450 nm–490 nm bandwith) and is equipped with a motorized precision stage (of up to 5 slides) and a CCD color camera. All images were acquired using a 40x objective lens. Software was developed to perform automatic reading and interpretation of Hep-2 cells.

To perform the reading step, the system manages an image mosaicing technique by using known translations of the motorized stage. The mosaic of the overall well area is composed by stitching the images according to the known relative positions. At the end of the process, the image is used as a virtual microscope, viewing any detail of the digitalized well at different digital zooms. To acquire the image within focus, two steps are performed: a regular grid of focus points is defined over the scanning area and a camera auto-regulation technique is carried out before auto-focusing in each point. During the auto-regulation procedure, camera parameters, gain and exposure time are changed such that the image appearance (intensity) is comparable between positive, weakly positive, and negative samples. This allows for a successful focus procedure without having to rely on an additional counter stain.

The positive/negative discrimination is evaluated while the system is focusing over cells. The sensitivity of the camera is regulated over the entire image in order to reach a cell target density. The system was trained over a collection of sera in order to discriminate a positive or negative sample by evaluation of the parameters that regulate the sensitivity of the camera acquisition. A probability measure of positivity (probability index) is calculated based on statistics of a set of training samples. This probability index is used to classify the sample in positive, negative or uncertain.

The system performs intelligent pattern recognition (five patterns can be assigned: homogeneous, nucleolar, speckled, centromere and mitochondrial). The first step in the image processing algorithm is the use of morphological operators and threshold techniques to separate background from foreground. The segmentation of foreground is performed in order to evaluate single cells. In a second step, texture features are calculated that evaluate the intensity surface of the cells. Finally, a supervised learning classifier is used to classify patterns by using the descriptors.

All samples were analyzed at dilution 1:80. Sample dilution was performed by Zenit-SP automate (A. Menarini Diagnostics).

Two substrates were used for analysis: HEp-2 (A. Menarini Diagnostics) and HEp-2000 (Immunoconcepts Sacramento, CA, USA). The conjugate used was IgG specific for the HEp-2000 substrate and IgG (heavy and light) for the HEp-2 substrate. Samples for which the fluorescence intensity did not reflect the reactivity of the autoantibody to the substrate (as evidenced by visual inspection of the digital image) were excluded (presence of artifact or bad quality of the sample).

In preliminary experiments we confirmed adequate acquisition of images by G-Sight (by using the first cohort). G-Sight detected antibodies to centriole (n = 3), centromere (n = 37), the cytoplasm (fibrillar pattern) (n = 7), membrane (n = 14), midbody (n = 8), proliferating cell nuclear antigen (n = 8), mitotic spindle (n = 16) (one sample was missed), Scl-70 (39), RNP (n = 26), SSA (n = 48) (3 samples were missed on HEp-2000 and 8 were missed on HEp-2.

A first set of controls [homogenous pattern] was run during analysis of cohort 1 and cohort 2, whereas a second set of controls [homogenous pattern] was run during analysis of cohort 3. With the HEp-2 substrate, the coefficient of variation (CV) was 8.9% for the first positive control (mean 67.6; range 59–78) (n = 12), 3.1% for the second positive control (mean 95.8; range 86.9-97) (n = 11) and 79.9% for the second low control (mean 14.6; range 0–24.5) (n = 11). The first low control had a value of 0 for all determinations (n = 12). With the HEp-2000 substrate, the CV was 0.8% for the first positive control (mean 89.5; range 88–90) (n = 11), 1.4% for the second positive control (mean 88.8; range 86.4-90.2) (n = 13), 60% for the first low control (mean value 15.4; range 4–32) (n = 11), and 60.7% for the second low control (mean value 41.9; range 9.8-80.8) (n = 12).

3. Results

3.1. Image acquisition by G-Sight

Automated antinuclear antibody analysis by G-Sight was performed on 268 consecutive samples submitted to the laboratory. The analysis

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