Journal of Immunological Methods 404 (2014) 52–58

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Research paper

Novel method for ANA quantitation using IIF imaging system

Xiaodong Peng *, Jiangtao Tang [1], Yongkang Wu, Bin Yang, Jing Hu

Department of Laboratory Medicine, West China Hospital, Sichuan University, Chengdu 610041, China

ARTICLE INFO

Article history:
Received 17 August 2013
Received in revised form 18 November 2013
Accepted 16 December 2013
Available online 23 December 2013

Keywords:
Antinuclear antibodies (ANAs)
HEp-2 cells
IIF image analysis system
Quantitative measurement

ABSTRACT

A variety of antinuclear antibodies (ANAs) are found in the serum of patients with autoimmune diseases. The detection of abnormal ANA titers is a critical criterion for diagnosis of systemic lupus erythematosus (SLE) and other connective tissue diseases. Indirect immunofluorescence assay (IIF) on HEp-2 cells is the gold standard method to determine the presence of ANA and therefore provides information about the localization of autoantigens that are useful for diagnosis. However, its utility was limited in prognosing and monitoring of disease activity due to the lack of standardization in performing the technique, subjectivity in interpreting the results and the fact that it is only semi-quantitative. On the other hand, ELISA for the detection of ANA can quantitate ANA but could not provide further information about the localization of the autoantigens. It would be ideal to integrate both of the quantitative and qualitative methods. To address this issue, this study was conducted to quantitatively detect ANAs by using IIF imaging analysis system. Serum samples from patients with ANA positive (including speckled, homogeneous, nuclear mixture and cytoplasmic mixture patterns) and negative were detected for ANA titers by the classical IIF and analyzed by an image system, the image of each sample was acquired by the digital imaging system and the green fluorescence intensity was quantified by the Image-Pro plus software. A good correlation was found in between two methods and the correlation coefficients (R^2) of various ANA patterns were 0.942 (speckled), 0.942 (homogeneous), 0.923 (nuclear mixture) and 0.760 (cytoplasmic mixture), respectively. The fluorescence density was linearly correlated with the log of ANA titers in various ANA patterns ($R^2 > 0.95$). Moreover, the novel ANA quantitation method showed good reproducibility ($F = 0.091$, $p > 0.05$) with mean ± SD and CV% of positive, and negative quality controls were equal to 126.4 ± 9.6 and 7.6%, 10.4 ± 1.25 and 12.0%, respectively. In conclusion, our novel ANA quantitation method can provide both of the fluorescence density, which could precisely reflect the fluctuate of ANAs level in patient's serum and the useful information about the localization of the autoantigens for clinician in diagnosing and monitoring diseases.

1. Introduction

A variety of antinuclear antibodies (ANAs) are found in patients with a number of autoimmune diseases. The detection and quantitation of ANAs is pivotal to the diagnosis of many autoimmune disease (Solomon et al., 2002; Agmon-Levin et al., 2010). Among others, the frequency of ANA is much higher in patients with systemic lupus erythematosus (SLE), mixed connective tissue disease, Sjögren's syndrome, scleroderma and other connective tissue diseases (Kavanaugh et al., 2000; Sinico et al., 2002). The degree of ANA positivity is diagnostically important in SLE since the positive predictive value increases with high titers (Pham et al., 2005). Hence, it was internationally recommended that the detection of ANA is the first level for laboratory diagnosis of systemic autoimmune-rheumatic diseases (SARD) (Agmon-Levin et al., 2013).

Indirect immunofluorescence assay (IIF) on HEp-2 cells remains the golden standard method for screening of ANAs and commonly used in most laboratories in the world to date (Tozzoli et al., 2002; Invernizzi et al., 2007; Meroni and

* Corresponding author. Tel.: +86 28 85422752.
E-mail address: pxd@scu.edu.cn (X. Peng).
[1] Co-first author.

0022-1759/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jim.2013.12.004

Schur, 2010). IIF can determine the presence of ANAs and provide information about the localization of the autoantigens. The staining patterns of ANAs, which include homogeneous, speckled, centromere, nucleolar, cytoplasmic and other mixed patterns, etc., could provide useful information for clinicians to diagnose diseases because of some patterns are associated with certain autoantigen and/or autoimmune diseases (Tan, 1989; Agmon-Levin et al., 2013; Mariz et al., 2011). However, the utility of ANA titer detected by IIF in diagnosing, prognosing and monitoring of disease activity has been limited due to the lack of standardization in performing the technique, subjectivity in interpreting the results and the fact that it is only semi-quantitative.

Numerous enzyme immunoassays have been developed as alternative methods for ANA screening in an attempt to make the method more objective and less labor intensive. For example, enzyme-linked immunosorbent assay (ELISA) is a quantitative method for ANAs detection and is more convenient for large number of samples than IIF method. But it may vary in the specificity and/or purity of antigenic epitopes, concentration and binding capacity of antigens, and the secondary antibodies prepared. It could not provide further information about the localization of the autoantigens aid in the diagnosis of disease. It is only able to detect a limited number of ANAs and may result in false negative (Anon, 2009). Therefore, to date, none of enzyme immunoassays has been widely accepted as an alternative to IIF for detection and titration of ANAs (Hiepe et al., 2000; Nifli et al., 2006).

It would be optimal to integrate the advantage of the qualitative and quantitative methods mentioned above. Recently, some researchers have attempted to make the classical IIF method more objective and less labor intensive. The use of digital images of HEp-2 cell testing was found to be reliable and helpful in determining ANA titers. It is more objective and could reduce the variation among different laboratories and even among different observers in the same laboratory (Peterson et al., 2009; Peng et al., 2002). However, the amount of ANAs is still reported as endpoint titers which are only semi-quantitative measurement.

In this study, we established a really quantitative method for measurement of ANA levels in patient sera by using a combination of IIF method (the classic standard method), a digital imaging system and an image analysis system.

2. Materials and methods

2.1. Serum samples and reagents

Serum samples were obtained from in- and out-patients who submitted for ANA-IIF testing at the Clinical Immunology Laboratory of West China Hospital, Sichuan University, China. 51 ANA-IIF negative and 1699 ANA-IIF positive samples with various fluorescence patterns (412 speckled, 300 homogeneous, 542 nuclear mixture and 445 cytoplasmic mixture) were analyzed as shown in Table 1. Positive and negative ANA controls provided by ANA test kits (EUROIMMUN Inc., Germany) were tested in parallel with patient sera. The instruments used in this study include fluorescent microscope (Nikon EC600, Japan), the digital Imaging System (SPOT32, American) and the image analysis system (Image-Pro plus software ipwin32, American).

Table 1
Serum samples from patients with various pattern and titer of ANAs (n).

Pattern	Total number	Number of serial ANA titer				
		1:100	1:320	1:1000	1:3200	1:10000
Speckled	412	50	98	139	69	56
Homogeneous	300	50	66	75	55	54
Nuclear mixture[a]	542	50	132	234	71	55
Cytoplasmic mixture[b]	445	52	81	205	54	53
Negative	51					

[a] Mixture of speckled/homogeneous and other patterns.
[b] Mixture of cytoplasmic and/or centromere/nucleolar/dots.

2.2. Detection of ANA titer by indirect immunofluorescence assay (IIF)

Serum samples and quality controls were detected for ANAs titer by IIF with ANA test kit according to the manufacturer's instruction (EUROIMMUN Inc., Germany). In brief, serum samples were performed a dilution series (1:100, 1:320, 1:1000, 1:3200 and 1:10000) in phosphate-buffered saline (PBS). Diluted serum samples and quality controls were applied to HEp-2 cells on slides and incubated for 30 min at room temperature. Then the slides were flushed and immersed in PBS for about five minutes. After another incubation with secondary FITC-conjugated antibody for 30 min at room temperature, the slides were washed again and covered by glycerol. Each slide was observed under fluorescence microscope (amplification, 400×) and the highest dilution of serum to stain HEp-2 cells is reported as the endpoint titer of ANA.

2.3. Quantitation of ANA by the Image analysis system

For quantitative determination of ANAs, the IIF images of each slide were acquired by a digital Imaging System (SPOT32, American), and the green fluorescence density of each image was analyzed by an image analysis system (Image-Pro plus software, ipwin32, American). Briefly, the serum samples from patients were performed by IIF as described above. Sera dilution at 1:100 was chosen for screening ANAs based on the recommend screening dilution from the manufacturer's instruction (EUROIMMUN Inc., Germany) as well as reports of a decreased percentage of false-positive results in healthy individuals (Rigon et al., 2007; Ghosh et al., 2007; Nifli et al., 2006; Dahle et al., 2004). The 4-s exposure time was chosen for taking each image, and the green fluorescence density of each image was measured in certain parameters (green density, area > 1000) (Fig. 1). Each sample was imaged and measured triplicate, and the average of three densities was reported as quantitative result of each sample. The quality controls provided in the kit were tested in parallel with sera samples.

2.4. Quality control of ANA by the Image analysis system

Quality control is very important and necessary in performing a quantitation assay. To control the quality of our quantitation method for ANA detection, 5 positive and 5 negative standards with the same Lot number provided in ANA