Evaluation of the Automatic Fluorescent Image Analyzer, Image Titer, for Quantitative Analysis of Antinuclear Antibodies

Tetsuo Nakabayashi,1 Toshiko Kumagai, PhD,1 Kazuyoshi Yamauchi, MS,1 Mitsutoshi Sugano,1 Akane Kuramoto,1 Kiyotaka Fujita, PhD,2 Hiroya Hidaka, PhD,1 and Minoru Tozuka, PhD1

Key Words: Antinuclear antibodies; ANA; Indirect immunofluorescent antinuclear antibody; FANA; Automatic fluorescent image analyzer; HEp-2 cultured cell; Autoimmune disease

Abstract

By making comparisons with the usual manual method, we evaluated an automatic fluorescent image analyzer (Image Titer, Tripath Imaging, Burlington NC), the software for which was developed to simplify measuring indirect immunofluorescent antinuclear antibodies (FANAs). In this new system, images of the stained sample are displayed, and it measures the FANA titer and staining pattern using only 1 slide per subject and does not required the staining of a series of diluted samples as does the manual method. This system showed good reproducibility and linearity for 4 types of control serum samples (with homogeneous, speckled, discrete speckled, and nucleolar staining patterns). In 132 serum samples, consistency between the methods was 100% for the FANA staining pattern and 93.9% for the FANA titer. The Image Titer system detected each pattern in samples with 2 mixed patterns. This system should partly reduce labor and lead to results with minimum differences among individuals, including newly trained persons.

A variety of antinuclear antibodies (ANAs) are found in serum samples obtained from patients with autoimmune diseases. Identification of the corresponding antigens and measurement of the antibody titer are useful for diagnosis and to inform about the probable progress and prognosis of the disease.1-3 At present, with HEp-2 cultured cells being used as a substrate, the indirect immunofluorescent antinuclear antibody (FANA) test is in use in many facilities for the measurement of ANAs. The FANA test permits determination of the corresponding antibodies by classification of staining types and has the advantage of permitting detection of a great variety of ANAs.4,5 Therefore, it is considered a useful primary screening test for the identification of ANAs. On the other hand, the FANA test is not convenient for the treatment of a large number of samples since the assay of antibody titer by this test requires a complex dilution procedure.6

Enzyme-linked immunosorbent assay (ELISA) FANA tests have been developed as routine screening tests. The method is convenient for large number of samples; however, a classification of staining types is not obtained.7-11

An automatic fluorescent image analyzer, Image Titer (manufactured by Tripath Imaging, Burlington NC, and sold by Medical & Biological Laboratories [MBL], Ina, Japan) was developed to simplify FANA quantitative analysis. On the basis of a single stained slide of a sample, the instrument displays images of stained samples at various dilution points on a monitor screen without the user having to stain a series of diluted samples. Since 1 sample requires only 1 well for quantitative measurement, this method should partly reduce labor.

We evaluated the Image Titer analyzer to assess its clinical usefulness for detecting FANA.
Materials and Methods

Serum Samples

We obtained 132 serum samples from patients with various ANAs (as indicated by the manual FANA method).

Slide Preparation

For the Image Titer system, slides to be used to construct a standard curve were prepared as follows: Eight levels of standard sample were prepared by stepwise dilution of a well-characterized calibration standard for ANA, a high titer (1:5,120) homogeneous pattern serum included in the Fluoro HEPANA Test for Image Titer (MBL) as one of the reagents, using a diluting solution (phosphate-buffered saline [PBS] containing 4% bovine serum albumin, pH 7.4, MBL). The concentrations of the standard are described as FANA titers 40, 80, 160, 320, 640, 1,280, 2,560, and 5,120. Slides (Fluoro HEPANA Test) carrying HEp-2 cultured cells were incubated with the standard samples for 30 minutes at 37 C and then washed with PBS twice and incubated for 30 minutes at 37 C with fluorescein isothiocyanate–conjugated antihuman immunoglobulins (MBL). They were washed twice again and observed under a fluorescence microscope (BX-60, Olympus, Tokyo, Japan). Slides incubated with the patient’s serum samples diluted to 1:40 with the diluting solution also were stained as described.

For the manual FANA method, each of the patient’s serum samples diluted stepwise with PBS was incubated with a slide, followed by staining as described.

Image Titer

The Image Titer setup is shown in **Figure 1**. The software provided with our system, which was developed to simplify the measurement of FANA, was compatible with MS-Windows 3.1 but has since been upgraded to compatibility with MS-Windows NT (Microsoft, Redmond, WA).

Principles of the Image Titer System

Standard Curve

Each standard slide is prepared as described. From its FANA staining image, a typical field is selected under the microscope. The image is delivered to the Image Titer software via a charge-coupled device (CCD) camera mounted on the trinocular head of the microscope. The software then displays 32 images for each standard. These 32 images are prepared by changing the exposure time and contrast strength in the CCD camera. Exposure time is changed in 8 steps, from 1 to 1/128 sec, and contrast conditions are changed in 4 steps for each exposure time. There is no need for concern that fluorescence fades, since a light irradiates only 2.008 seconds for an analysis on 1 field. From the 32 images, the operator selects one as an endpoint. The exposure time and contrast strength are converted to a number called the image endpoint (IE), where IE = 1/exposure time (seconds) + contrast strength (from 0.2 to 0.8). Then, the data for IE and the concentration (with respect to the standard) are recorded to produce a standard curve. The standard curve is corrected by use of a built-in best-fit curve.

Analysis of Serum Samples

The FANA staining pattern of a prepared slide is determined under the microscope by the operator. The image is delivered to the Image Titer, and then the software displays 8 images using data from the previously established standard curve. The operator selects the endpoint and records the pattern.

Results

Reproducibility

The reproducibility of the Image Titer system for measuring FANA titer and pattern was assessed using 4 types of control serum samples. No significant difference was observed in the results among 5 fields, the center, and 4 fields around the center (data not shown). FANA titer values were obtained 8 times within each run, and values also were
obtained on 5 consecutive days (data not shown). Good reproducibility for the FANA titer in all staining patterns (within ±1 dilution) was obtained for within- and between-run precision. No significant difference was observed in the reproducibility among the different people using the Image Titer system (data not shown). We also compared the results obtained by the different people using the manual method and the Image Titer system, including a well-trained, a newly trained, and an inexperienced person. Compared with the manual method, Image Titer system gave results with smaller variations among individuals.

**Linearly**

High titer samples for the 4 staining patterns (nucleolar, discrete speckled, homogeneous, and speckled patterns) were diluted stepwise with diluting solution, and linearity was examined. For each of the 4 staining patterns, the titer decreased linearly as the dilution ratio increased. For each sample, all points but one were within the permissible range either side of the theoretical value (ie, within ±1 dilution, with 1:40 as the starting dilution point). Only 1 point for the sample with the discrete speckled pattern was out of range (at 1:320 dilution).

**Correlation Between the Image Titer and Manual Methods**

Of the 132 serum samples tested, all showed the same FANA staining pattern by the Image Titer as by the manual method. There was a fine correlation between both methods. In 124 (93.9%) of 132 samples, the FANA titer value obtained using the Image Titer coincided with the titer obtained by the manual method with sufficient accuracy (ie, within ±1 dilution). Of 132 samples, 8 were out of range: 1 sample with the homogeneous pattern and 3 samples with the speckled pattern were 2 dilutions higher than by the manual method, and 1 each with the homogeneous and discrete speckled pattern and 2 with the speckled pattern were 2 dilutions lower than by the manual method. By
Studies on Samples With Mixed Patterns

Image Titer creates images of samples diluted stepwise by extrapolation from a slide at 1 dilution point, and a staining pattern with a higher titer may hide one with a lower titer when 2 or more antibodies are mixed in 1 sample. So, we used samples containing multiple antibodies and compared images obtained from the Image Titer with those from the manual dilution method. **Image 1A** shows images of a sample containing homogeneous and nucleolar staining patterns by the 2 methods. At the 1:40 dilution point, the nucleolar pattern was masked by the homogeneous pattern in both methods. As the dilution increased from 1:80 to 1:320, the nucleolar pattern was identified clearly by both methods, but it was less clear at the 1:80 dilution point by the Image Titer system than by the manual method. **Image 1B** shows a sample containing a proliferating cell nuclear antigen and a homogeneous mixed pattern. Both methods enabled us to identify the proliferating cell nuclear antigen pattern at the 1:160 dilution point.

Discussion

The results of the present study indicate that the Image Titer system is as sensitive and specific as the widely used FANA manual measuring method, and it should partly reduce labor and lead to results with minimum differences among individuals. Measurements of FANA titers and staining patterns by this system showed good reproducibility, linearity, and correlation with the manual method. In the manual method, quantitative analysis of antibody titer requires 2 to 4 slides per sample and a complex procedure. So, it is not suitable for the treatment of a large number of samples (especially positive samples). In contrast, only 1 slide per sample is needed with the Image Titer system.

With the Image Titer system, nonspecific staining was minimal, and we could determine the pattern and endpoint of...
FANA using a special diluting solution with bovine serum albumin, even though the possibility of a nonspecific result was taken into consideration because the images were based on a sample with a low dilution ratio (1:40). Several ELISAs for large scale screening of ANAs using recombinant and purified nuclear antigens, or nuclear extracts, have been described.7-11 The Image Titer system has several advantages over ELISA methods. The most important is that the Image Titer system can provide images of a variety of ANAs, making it easy to identify the corresponding nuclear antigen or organelle, such as DNA, extractable nuclear antigen, nucleolus, centromere, and mitochondria. Such antigen-antibody systems are closely linked with collagen diseases, autoimmune hepatitis, primary biliary cirrhosis, and other autoimmune diseases. Identification of FANAs makes an important contribution to the diagnosis of autoimmune diseases.

FANA staining patterns reflect the distribution of the corresponding nuclear antigen, and so the patterns show many variations. For example, the patterns of anti-Sm, U1-RNP,17 Ro/SSA, and La/SSB18 antibodies are all classified as speckled, but the distributions of their corresponding antigens show subtle differences. The nucleolar pattern is classified into 3 additional patterns according to the presence of distinct zones in the nucleolus, the granular component, the dense fibrillar component, or the fibrillar center. These complex patterns make it difficult to measure the FANA endpoint both by the manual method and by the Image Titer system. This is one of the major reasons for the slight differences in FANA titer between the two methods (as shown in Figure 4). The Image Titer system reduces the influence of this factor by preparing a standard curve.

When a sample contains more than one antibody, there is a possibility that the weaker staining pattern could be hidden. In such cases, the Image Titer system was as good at detecting the weaker pattern as the manual method (Images 1A and 1B). This is considered to reflect the usefulness of virtual images.
Conclusions

By the development of software for an automatic image analyzer, a simplified FANA quantitative analysis has been established. Use of this system for mass screening should partly reduce labor and the difference in results among individuals. It should be useful for the clinical study of autoimmune diseases.

References