Experimental Investigations

MICROMERITIC PROCEDURES IN ASSESSING ANTINUCLEAR ANTIBODY PATTERNS
IN IMMUNOFUORESCENT ASSAY

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ABSTRACT

**Aim:** The aim of this study was to introduce a micromeritic procedure (a statistical approach for small objects) in indirect immunofluorescence assay (IFA) to find objective quantitative parameters of antinuclear antibody (ANA) patterns which could support a diagnosis of auto-immune diseases. **Materials and methods:** Sera of patients with systemic autoimmune diseases, McCoy-Plovdiv serum-free cell line, goat anti-human immunoglobulin-G FITC-conjugate, fluorescent microscope, computer-assisted digital image processing, analysis using a micromeritic procedure, ANOVA. **Results:** Three ANA fluorescent patterns (homogeneous, rim and speckled) were analyzed by the micromeritic procedure. Parameters for the image brightness of the pixels (pixel grey value) were obtained and discussed as objective characteristics of fluorescent patterns: maximum ANA-linkage volume and surface density were established for the objects with speckled localization pattern. **Conclusion:** The micromeritic method for getting objective quantitative values of ANA fluorescent patterns in indirect immunofluorescent assay might be a valuable tool aiding in immunological diagnosing if integrated in a laboratory software package.

**Key words:** antinuclear antibodies, immunofluorescence, image analysis, FITC, pixel value distribution

**ЗАКЛЮЧЕНИЕ**

ЦЕЛЬ: Работа ставит себе целью ввести микрометрическую процедуру (статистический анализ, применимый для небольших объектов) в индиректный флюоресцентный анализ в целях получения объективных количественных параметров при анализе локализации антинуклеарных антител в диагностике автоиммунных заболеваний. **Материалы и методы:** Применены: сыворотки пациентов с системными автоиммунными заболеваниями; безъядрочная клеточная линия McCoy-Plovdiv; маркированный флюорохромом FITC иммуноглобулин; флюоресцентный микроскоп; цифровизирование и обработка изображений; микрометрический анализ; ANOVA статистическая обработка данных. **Результаты:** Три вида локализации антитела (ANA) реакции - гомогенная, периферическая и петнистая - анализировались микрометрическим методом. Считается, что яркость пикселов флюоресцентных изображений служит объективной характеристикой вида локализации. Максимальный объем и поверхностная плотность связанных с субстратом ANA получены для петнистого типа локализации. **Заключение:** Микрометрический метод количественной характеристики типа ANA локализации в индиректной иммунофлюоресценции может оказаться ценным дополнительным инструментом для анализа в иммунологических диагностических лабораториях с возможностью для включения в пакет программного обеспечения (software) компьютерной программы для обработки изображений.

**Ключевые слова:** антинуклеарные антитела, иммунофлюоресценция, цифровой анализ изображений, FITC, распределение размера пикселя

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INTRODUCTION

Antinuclear antibodies (ANA) are directed against various nuclear antigens, form complexes with them, and cause tissue damage in systemic lupus erythematosus and other non-organ specific autoimmune diseases. Indirect immunofluorescence assay (IFA) is the standard immunological test to screen ANA presence in sera of affected patients. Nuclear antigens, reacting with serum ANA, can be determined by secondary fluorochrome-conjugated anti-immunoglobulin antibodies. Fluorescein isothiocyanate (FITC) is the most widely used fluorochrome. The detected fluorescent patterns vary as homogeneous (diffuse), peripheral (rim), speckled, nucleolar, centromere, etc. They correlate with different localization of antigen-antibody reaction and help to identify fine specificities associated with a particular disease or clinical manifestations. Detection of fluorescent type and intensity are highly dependent on observer’s qualification. Various procedures are used to improve the quality of ANA readings through sera screening dilution, substrates and microscope technique. The introduction of digital images and computer-assisted systems in IFA practice is a promising development that may help to achieve a more objective assessment of ANA patterns.

AIM

The main goal of this study is to introduce the micromeritic procedure for obtaining objective quantitative parameters which could support fluorescent ANA determination and hence the diagnosis of auto-immune diseases.

MATERIALS AND METHODS

IFA TESTING

Procedures. The IFA of ANA testing from patients’ sera was performed as described previously using McCoy-Plovdiv serum-free cell line as a substrate for ANA detection. Ready-to-use goat anti-human immunoglobulin G FITC-conjugate (Binding Site, UK) was used as a secondary antibody to visualize antigen-antibody reaction. The conjugate binds to those serum ANA immunoglobulins that react with the nuclear antigens of the cell line.

Microscope examination of staining patterns. Slides prepared as previously described were examined on a fluorescent microscope (Optiphot 2, Nikon Corp., Japan) at 400x magnification to determine the staining pattern with planapochromate objective lens – 40x. The reaction between ANA and nuclear antigens of McCoy-Plovdiv cells was visualized as apple-green staining of nuclear structures under UV light. The intensity and patterns of fluorescence were assessed and recorded.

Selection of fluorescent patterns for analysis. Three nuclear fluorescent patterns, corresponding to different ANA localization in the nucleus were selected: homogenous (H) – diffuse staining of the interphase nuclei and staining of the chromatin of mitotic cells; rim (R) – peripheral solid staining around the outer region of the nucleus with weaker staining toward the center of the nucleus; speckled (S) – a fine or course granular nuclear staining of the interphase cell nuclei.

Sera with subjectively assessed maximum fluorescent intensity (4+) were used for further analysis. Control sera from healthy individuals, negative for ANA, were used in the study. The non-specific fluorescent background, obtained in the negative controls, was subtracted from the fluorescent staining of ANA positive sera.

DIGITAL IMAGE PROCESSING AND ANALYSIS

The representative patterns were acquired by an operator with a charge-coupled device (CCD) camera Panasonic and transferred to a personal computer. The RGB color image was converted to a grey scale and 20 single objects (nuclei) were extracted from the image frames with different types of localizations. The brightness (grey level value) of the pixels were obtained from the line density plot profiles of the selected single nucleus by image software (Scion Image 4.0.32, Maryland, USA).

The pixel grey value (PGV) distributions on a number basis (A) and on a weight basis (B) were obtained using the micromeritic procedure described elsewhere at different staining patterns. Distribution A corresponds to the number of pixels observed within a selected grey value range (Y-axis) plotted against mean PGV for ranges (X-axis). Distribution B is acquired from distribution A after calculation of the weight of the pixels. The number of pixels, having selected grey value or weight, are presented as percentage of the total number of pixels.

Micromeritic parameters from distributions A and B (length, surface, volume, density) are calculated using the corresponding formulae.

STATISTICAL ANALYSIS

All measured values were validated and entered into a database for further analysis. Data were
statistically analyzed by descriptive statistics (mean ± SD values provided in the results) and one-way ANOVA. The ANOVA post hoc multiple comparison analysis were performed with the Tukey’s test in order to find which mean values are significantly different from one another. Statistical significance was set at p < 0.05. All statistical analyses were performed using SPSS v. 13.

RESULTS AND DISCUSSION

The microscope images of the sera of three patients with different autoimmune diseases are presented in Fig. 1. The figure shows different patterns of fluorescent nuclear localization - homogeneous (H), rim (R) and speckled (S). These images correspond to (4+) semi-quantitative scale of ANA-sera patterns because this fluorescence intensity is easier for micromeritic testing.

Data for PGV obtained after line density plot profile procedure were statistically analyzed and are presented in Table 1. The mean PGV among H, R and S localization differ significantly between S (58.84 ± 2.50) and H (87.79 ± 2.81), as well as between S (58.84 ± 2.50) and R (78.42 ± 4.60) localizations (F=19.85, p < 0.001).

Cumulative frequency plots with Distributions A (on a number basis) and B (on a weight basis) of PGV are shown in Fig. 2. Both distributions for the same samples put an emphasis on different asymmetry level of the antigenic nuclear object in terms of micromeritic parameters (length, surface, volume, density). These parameters are summarized in Table 2.

The data in Table 2 further illustrate the importance of both distributions for better characterization of the digital image properties as a result of micromeritic approach:

• the average PGV, calculated from the micromeritic parameter volume (Distribution A) of the antigen in the nucleus, has a minimum value for speckled localization pattern – 15.88 vs 113.99 (for H) and 94.71 (for R). This minimum value corresponds to maximum volume density for ANA-linkage to nuclear antigens11;

• the average PGV, calculated from the micromeritic parameter surface (Distribution B) has a minimum value again for speckled localization pattern – 75.07 vs 139.88 (for H) and 110.82 (for R). This minimum value is inversely proportional to the real surface11 of the nuclear antigens for ANA linkage and is, probably, an indication for maximum surface capacity of the nuclear antigens for ANA linkage.

In this study we present quantitative parameters for objective determination of ANA fluorescent patterns by micromeritics – a statistical approach for small objects.8,11,12 The fluorescent intensity (brightness) data are represented by PGV for three distinct ANA fluorescent patterns – homogenous,

| Table 1. Mean PGV of H, R and S-localizations of ANA |
|-----------------|-----------------|-----------------|
| Descriptive statistics | Localization |
| | H | R | S |
| N | 366 | 363 | 409 |
| MEAN | 87.79 | 78.42 | 58.84* |
| SD | 53.68 | 87.56 | 50.56 |
| SEM | 2.81 | 4.60 | 2.50 |

*statistically significant values (p < 0.001) for S vs. H or R localizations.
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Table 2. Average PGV calculated from Number distributions A and Weight distributions B

<table>
<thead>
<tr>
<th>Micromeritics parameters</th>
<th>Average PGV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H-localization</td>
</tr>
<tr>
<td>From distribution A</td>
<td></td>
</tr>
<tr>
<td>length</td>
<td>87.80</td>
</tr>
<tr>
<td>surface</td>
<td>102.90</td>
</tr>
<tr>
<td>volume</td>
<td>113.99</td>
</tr>
<tr>
<td>volume density(^a)</td>
<td>~ 1/113.99</td>
</tr>
<tr>
<td>From distribution B</td>
<td></td>
</tr>
<tr>
<td>length</td>
<td>120.61</td>
</tr>
<tr>
<td>surface</td>
<td>139.88</td>
</tr>
<tr>
<td>surface density(^b)</td>
<td>~ 1/139.88</td>
</tr>
<tr>
<td>volume</td>
<td>151.77</td>
</tr>
</tbody>
</table>

\(^a\) volume density ~ 1/ volume; \(^b\) surface density ~ 1/surface;
\(^*\) statistically significant difference (p < 0.01).

rim and speckled. Homogeneous nuclear staining is a result of immune reaction between ANA and DNA in the nucleus; rim pattern appears in older cell cultures and represents ANA linkage to DNA too, whereas speckled pattern reflects reaction between ANA and extractable nuclear antigens (SS-A, SS-B, Sm, RNP).

There have been a number of attempts to avoid subjective assessment of microscopic images in ANA readings by IFA. They focus either on sample preparation and adjustment of microscopes or applying computer-assisted approaches with image processing and analysis.\(^6,7,10,13\) Nevertheless, the relevant literature has little data about the positive aspects of micromeritic method and exploration of parameters in the field of ANA detection which have been introduced in the present study. The objective quantitative criteria used in this method could assist inexperienced observer in ANA detection pattern, especially in the differentiation between speckled fluorescence, on the one hand, and homogeneous and rim fluorescence, on the other.

**CONCLUSIONS**

In conclusion, the obtained results suggest that the micromeritic method can be used to achieve objective quantification of ANA fluorescent patterns in indirect immunofluorescent assay. This approach supports the IFA test and is a valuable additional tool to immunological laboratory diagnostics, suitable to be included in software packages.

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Distribution A is calculated from PGV on a number basis; Distribution B is received from the same pixels on a “weight” basis.

**Figure 2.** Cumulative frequency plots for three ANA patterns of localization.
REFERENCES


