A COMPARATIVE STUDY OF IMMUNOLOGICAL METHODS FOR DETERMINATION OF SERUM ANTINUCLEAR ANTIBODIES

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ABSTRACT

INTRODUCTION: Several immunological methods are used to determine the serum antinuclear antibodies (ANA). Indirect immunofluorescence assay (IFA) with tissue slices or HEp-2 cells is the standard technique considered the gold standard for their screening. Serum-free McCoy-Plovdiv cell line may also be used as substrate for IFA. Another method for detection of total and specific ANA is the enzyme-linked immunosorbent assay (ELISA). Immunoblot is also applied in specific ANA confirmation.

The aim of the current study was to determine and propose a justified immunological approach for identification of clinically significant ANA by comparing the screening tests - ANA-IFA on serum-free McCoy-Plovdiv cell substrate with ELISA for total ANA, and confirmative methods for specific ANA – ELISA with immunoblot.

MATERIALS AND METHODS: Serum samples from 38 patients screened for total ANA by ELISA (Trinity Biotech, NY, USA) and IFA-ANA with McCoy-Plovdiv cell line, were included in the study. Positive samples were confirmed by immunoblot (Orgentec Diagnostika, Germany) and ELISA for specificity of confirmed ANA.

RESULTS: No significant difference (P > 0.05) and very good agreement were found between the two screening tests. Very good agreement for specific antibodies against SS-A, SS-B, dsDNA, moderate for anti-Sm and anti-Sm/RNP and fair for anti-histone/nucleosomal antibodies was found between confirmative methods. No agreement was found for anti-Scl-70 antibodies.

CONCLUSION: IFA-ANA with serum-free McCoy-Plovdiv cell line and screening ELISA may be recommended for determination of total ANA, and immunoblot and ELISA – for confirmation and identification of specific ANA.

Key words: antinuclear antibodies, indirect immunofluorescence, enzyme-linked immunosorbent assay, immunoblot, McCoy-Plovdiv cellular line

INTRODUCTION

Detection of antinuclear antibodies in serum is used in the diagnostics of systemic lupus erythematosus (SLE) and other connective tissue autoimmune disorders.¹² Different methods are applied for their measurement, but indirect immunofluorescence assay (IFA) has remained the “gold standard” in ANA screening for more than 50 years.³⁵ Initially, rodent tissue samples (rat liver) were used as test substrates, but later human epithelial cell line HEp-2 and its modification HEp-2000 were introduced into routine practice, increasing IFA sensitivity for ANA detection.⁶⁷ McCoy-Plovdiv cell line is also used as an alternative substrate in ANA diagnostics.⁸⁹ Different patterns of nuclear fluorescence – homogenous, speckled (fine and coarse), centromere, nucleolar, SS-A, associated with antibodies against specific nuclear antigens and typical for different autoimmune disorders, are found on IFA-ANA.⁹¹⁰ Another widely used method
for ANA measurement is ELISA. It is automated, standardized, and relatively more convenient for screening great numbers of serum samples for ANA.

Specificity of ANA found with screening methods may be determined with the Farr test, counter immunoelectrophoresis, passive hemagglutination, IFA with *Crithidia lucilae* for anti-dsDNA, ELISA, immunoblot (linear immune test), multiplex immunotest, flow cytometry, and antigen microarray.\(^1,2,11-13\) The latter two techniques are still experimental, while ELISA and immunoblot are regularly used in clinical practice. Clinically significant autoantibodies against dsDNA, histones, SS-A, SS-B, Sm, RNP, Jo-1, Scl-70 are specified using these tests – with purified nuclear extracts and recombinant antigens.\(^14,15\) The strategies for ANA determination differ from laboratory to laboratory. However, finding the most appropriate (sensitive, specific and economically justified) combination of methods is still a subject of intensive comparative studies. No clear algorithm for ANA diagnosis is present in laboratory immunology. Hence, the aim of the current study is to determine and propose a justified immunological approach for identification of clinically significant ANA by comparing the screening tests - ANA-IFA on serum-free McCoy-Plovdiv cell line with ELISA for total ANA, and confirmative methods for specific ANA-ELISA with immunoblot.

**MATERIALS AND METHODS**

**SERUM SAMPLES**

The study included serum samples from 38 patients with suspected autoimmune disorders admitted to the Research Immunology Center at Medical University–Plovdiv and/or in the German-Bulgarian Medical Diagnostic Laboratory “Synwest-K” – Plovdiv for ANA determination. All serum samples were screened with IFA and ELISA for total ANA and the positive ones were tested with ELISA and immunoblot to determine the specificity of detected autoantibodies.

**METHODS**

1. **IFA-ANA. IFA-ANA (IgG)**

   IFA-ANA. IFA-ANA (IgG) was performed on serum-free McCoy-Plovdiv cell line as substrate according to Zagorov et al.\(^9\) The serum samples were diluted 1:40 with phosphate-buffer solution for IFA-ANA screening. Positive samples were down-titrated to 1:1280 to determine the end-point positive ANA titer. Nuclear immunofluorescence was visually assessed by two independent experienced microscopists to avoid subjective bias in conclusion.

   No difference was found in the image assessment. Fluorescent signal detection was done by fluorescent microscope (Nikon Eclipse 80i, Japan) with 400 X magnification, and the image was photographed with a digital camera Nikon DS-5 Mc, Japan and stored automatically on computer for an eventual re-inspection. The intensity of each fluorescent image was interpreted as negative (-), weak (1+), moderate (2+), strong (3+) or very strong (4+), and the type – as homogenous, speckled, nucleolar, peripheral, SS-A.\(^2\) To avoid reduction in the fluorescent signal with time (the so called fluorescence „extinguishing“) determination of fluorescence model started immediately after the completion of the immune reaction within 1-2 min per sample with inspection of at least 5 visual fields.

2. **ELISA-ANA**

   2.1. ELISA Screen for total ANA (IgG) (Trinity Biotech, New York, USA) contains purified antigens from nuclear extracts of HEp-2 cells - dsDNA, histones, Sm, Sm/RNP, SS-A, SS-B, Scl-70, centromeres and Jo-1. Index values < 1 were accepted as negative, and ≥ 1 as positive.

   2.2 ELISA for determination of specific ANA (Trinity Biotech, New York, USA) contains purified nuclear antigens of the specific type. In case of homogenous fluorescence on IFA, the samples were tested for antibodies against dsDNA and histones, in case of speckled fluorescence – for ANA against extractable nuclear antigens Sm, Sm/RNP, SS-A, SS-B and Scl-70, and in case of nucleolar fluorescence - for Scl-70.\(^9,13,16\) Negative values were accepted for ANA-index ≤ 0.90, borderline – between 0.91 and 1.09, and positive ≥ 1.10. ELISA Screen and ELISA for specific ANA were performed according to the manufacturer’s instructions. The optical density of the sample was determined by an absorbance reader (TECAN SUNRISE, Austria) at 450 nm and 650 nm.

3. **IMMUNOBLOT FOR ANA**

   Nucleo-9-Line immunoblot (Orgentec Diagnostika, Germany) was applied for simultaneous detection of specific IgG ANA against dsDNA, nucleosomes, SS-A, SS-B, Sm, Sm/RNP, Scl-70, Jo-1 and centromeres (CENP-B). The presence of specific ANA was detected by scanning device with specialized software (iVision Scanware) after calibration, validation by internal serum, conjugate and cut-off controls, according to manufacturer’s recommendations. The result was presented graphically, and the reaction intensity, automatically measured by the software,
was objectively interpreted and semiquantitatively assessed by the program as negative (-), borderline (±), slightly positive (+), moderately positive (++) and strongly positive (+++) for ANA. Digitalized images of the immunoblot were saved and archived for re-analysis if necessary.

4. Statistical analysis
Agreement with respect to the final result (positive or negative ANA) between different methods was assessed by κ (kappa) coefficient of Cohen. Agreement was determined as poor in κ < 0.20; fair in κ = 0.21÷0.40; moderate in κ = 0.41÷0.60; good in κ = 0.61÷0.80 and very good in κ = 0.81÷1.00. Statistically significant difference was accepted at P < 0.05 (Fisher’s exact test). Data were analyzed with statistical software MedCalc, Version 8.1.0.0 (MedCalc Corp., Mariakerke, Belgium).

RESULTS
Comparison between IFA-ANA and ELISA for ANA screening
Positive samples in both tests were 15 out of a total of 38 (39%). Homogenous fluorescence (Fig. 1A) on IFA-ANA was observed in 5 (13%) serum samples, and speckled (Fig. 1B) – in 4 (10.5%). Mitochondrial cytoplasmic fluorescence (Fig. 1C) was found in 1 of 38 samples (2.6%), and SS-A type (Fig. 1D) in 2 (5%). Overlapping of different models of fluorescence was present in 3 serum samples (8%). In the first, homogenous and SS-A fluorescence were simultaneously present, in the second – homogenous and mitochondrial, and in the third – homogenous and speckled. The prevalent number of ANA-positive serum samples showed high titer ( > 1:160). According to their titer, ANA-positive serum samples were distributed as follows: 7 (18.4%) with final titer 1:1280, 2 (5.3%) – with 1:640, 3 (8%) – with 1:320, 1 (2.6%) – with 1:160, and 2 (5.3%) - with low ANA titer (1:40).

The comparison between the two methods with respect to their capacity for ANA screening showed very good agreement (κ = 0.890, confidence interval - CI 95%, 0.74÷1.000). No significant difference between the tests was found (P > 0.05).

Comparison between immunoblot and ELISA for specific ANA
Out of 15 serum samples that were ANA-positive at the screening tests, autoantibodies were confirmed and identified in 14 (93%) samples with ELISA and in 13 (87%) with immunoblot (P > 0.05). In one serum sample anticentromere (CENP-B) antibodies were detected with immunoblot, but ELISA was not available for their detection. One of the 15 ANA-positive serum samples on screening was negative...
Multiple ANA were found in most of the positive samples. Immunoblot testing determined ANA specific to 1 antigen in 6 serum samples, 2 ANA types were found in 2 samples, 3 ANA types – in 4 samples and 5 ANA types – in 1 sample. ELISA for specific ANA determined 6 serum samples with 1 ANA type, 5 – with 2 specific ANA, and 1 sample of each – with 4, 5 or 6 different ANA.

Comparison of immunoblot and ELISA for specific ANA showed very good agreement in determining the anti-SS-A (κ = 1.000, CI 95% 1.000÷1.000), SS-B (κ = 1.000, CI 95% 1.000÷1.000) and anti-dsDNA (κ = 0.825, CI 95% 0.589÷1.000), moderate for anti-Sm and anti-Sm/RNP (κ = 0.479, CI 95% 0.223÷1.000) and fair for anti-histone/nucleosomes ANA (κ = 0.303, CI 95% 0.000÷0.949) (Table 1).
Since anti-Scl-70 autoantibodies were detected only with ELISA and were not found with immunoblot, no correspondence was present between the two methods for these antibodies.

The correlation between the type of positive ANA on ELISA and the fluorescent pattern on IFA methods for ANA-detection stems from the great prevalence of connective tissue autoimmune disorders and the increased number of various ANA tests offered by different manufacturers. Their characteristics and features vary according to the type of included substrates, antigens and devices used. The importance of ANA as one of the basic criteria for diagnosis of SLE and other connective tissue disorders makes it essential that proper accurate, sensitive immunological tests for their determination be chosen. The three types of diagnostic methods for ANA detection (IFA, ELISA and immunoblot) we used are combined in a logical laboratory scheme (algorithm) representing a complex approach with significance not only in immunology, but also in scientific and practical medicine in general (Fig. 2). The combination of screening and confirmative test for ANA offers possibility for greater range of investigation and allows finding of ANA against different nuclear antigens. IFA with HEP-2 cell line is a sensitive screening method for ANA determination. However, the necessity of serum addition to the medium for cellular cultivation of was proven. Homogeneous nuclear fluorescence was found in all serum samples with presence of anti-dsDNA and anti-histone antibodies. Speckled fluorescence was confirmed in cases of proven extractable nuclear antigens - Sm, Sm/RNP, SS-A and SS-B. Antibodies against extractable nuclear antigens and anti-dsDNA antibodies were simultaneously found in cases of overlap of homogenous and speckled fluorescence.

**DISCUSSION**

The need for research on comparability of different methods for ANA-detection stems from the great prevalence of connective tissue autoimmune disorders and the increased number of various ANA tests offered by different manufacturers. Their characteristics and features vary according to the type of included substrates, antigens and devices used. The importance of ANA as one of the basic criteria for diagnosis of SLE and other connective tissue disorders makes it essential that proper accurate, sensitive immunological tests for their determination be chosen. The three types of diagnostic methods for ANA detection (IFA, ELISA and immunoblot) we used are combined in a logical laboratory scheme (algorithm) representing a complex approach with significance not only in immunology, but also in scientific and practical medicine in general (Fig. 2). The combination of screening and confirmative test for ANA offers possibility for greater range of investigation and allows finding of ANA against different nuclear antigens. IFA with HEP-2 cell line is a sensitive screening method for ANA determination. However, the necessity of serum addition to the medium for cellular cultivation of

Table 1. Immunoblot and ELISA for specific ANA in screening ANA-positive serum samples (n = 15)

<table>
<thead>
<tr>
<th>Methods</th>
<th>Serum samples, positive for specific ANA – number (n) and percentage (%)</th>
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<tbody>
<tr>
<td></td>
<td>anti-dsDNA</td>
</tr>
<tr>
<td>Immunoblot</td>
<td>7 (53.84%)*</td>
</tr>
<tr>
<td>ELISA</td>
<td>6 (42.85%)*</td>
</tr>
<tr>
<td>Kappa</td>
<td>0.825</td>
</tr>
<tr>
<td>95% CI</td>
<td>0.589±1.000</td>
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* - very good correspondence; ** - moderate correspondence; *** - fair agreement.

HEP-2 increases the test price. This drawback was overcome by the introduction of McCoy-Plovdiv serum-free cells to IFA. Our choice of IFA-ANA with McCoy-Plovdiv cells was motivated by previous encouraging studies conducted by our team, showing high sensitivity and specificity of the test with this original substrate. Consequently we believe that the proposed screening test for ANA with serum-free McCoy-Plovdiv cells enriches the bunch of present substrates for IFA, and its inclusion in the developed laboratory algorithm is an original contribution to the contemporary ANA diagnostics.
In the present study we found no statistically significant differences between ELISA and IFA with McCoy-Plovdiv cell substrate for determination of total ANA. However, we think IFA-ANA is superior to ELISA due to its feature of detecting different patterns of nuclear fluorescence, directing the choice of specific confirmative ELISA or immunoblot. Important advantage of IFA-ANA is the capability of finding antimitochondrial and other cytoplasmic antibodies, which is not possible with ELISA for total ANA. Therefore, we think that IFA-ANA is a very appropriate method for screening purposes.3,13

The limitation of the number of nuclear antigens in ELISA for ANA screening decreases the sensitivity of the test in comparison to IFA. In this respect Tozzoli et al. propose ANA screening with IFA.18 Unlike them, Bayer et al. prefer ELISA.16 Divate et al. report slightly higher sensitivity of ELISA – 84.3% (at cut-off > 1.0) and 88.2% (at cut-off > 2.0) in comparison with IFA – 82.4% (19). Summarizing literature data, Kumar et al. recommend ELISA for ANA screening to be used in cases of expected medium- and high-titer ANA, because the test may fail to detect positive low-titer ANA or some specific antinuclear antibodies not included in the substrate.2

Our data on the relation between the type of fluorescence and the specificity of detected ANA are consistent with the results from other studies finding association between antibodies against extractable nuclear antigens and speckled fluorescence on IFA, as well as between anti-dsDNA antibodies and homogenous fluorescence.13,15

ELISA presents the opportunity of qualitative assessment of ANA with different specificity, most often against dsDNA, histones, Sm, Sm/RNP, SS-A, SS-B and Scl-70. The sensitivity of the tests varies between 73% and 100%.20 This method is expensive due to the necessity of using several ELISA for detection of ANA against the respective nuclear antigens, unlike immunoblot, by which specific antigens are simultaneously determined on the membrane with immobilized nuclear antigens.

According to Phan et al. immunoblot is less sensitive in detecting anti-SS-A antibodies than ELISA, for example in Sjogren syndrome sensitivity is 70-85% for immunoblot, and 90-97% for ELISA; in SLE it is 10-15% and 33-60%, respectively.21 It is assumed that the lower sensitivity of immunoblot in anti-SS-A detection is due to “weak” transfer (blot) of SS-A antigen from the gel to the membrane, high non-specific background or the fact that 15% of anti-SS-A ANA recognize only conformational epitopes of SS-A 60 antigen – immunoblot, unlike ELISA, proves only linear epitopes.21,22 However, we found no difference between the two methods with respect to determination of these antibodies, probably because of the smaller number of investigated serum samples.

Figure 2. Recommended laboratory algorithm for ANA detection.
Denaturation of conformational determinants after separation of antigens with polyacrylamid-gel-electrophoresis (SDS-PAGE) in immunoblot leads to greater difficulties in finding ANA also against other extractable nuclear epitopes – U1 RNP, Scl-70, Sm, Jo-1. The use of blocking agents, for example detergent Tween 20, may cause renaturation of the antigen and may increase the intensity of weak strips from antigen-antibody reaction. The lower sensitivity of immunoblot for ANA against U1 RNP correlates with higher specificity, for example in mixed connective tissue disease immunoblot specificity is 65-75%, compared to 50-60% in ELISA. The specificity of ELISA for these ANA is increased with application of recombinant antigens.21 Our data confirm the lower sensitivity of immunoblot in comparison with ELISA for detection of ANA against Sm, Sm/RNP or Scl-70.2,21-23 We found no agreement between the two methods in detecting ANA against Scl-70, probably due to the small number of cases, but we had confirmed these specific antibodies with immunoblot in other investigated serum samples.

We found anti-nucleosomal ANA with immunoblot in 5 serum samples, and anti-histone ANA with ELISA in only one. The discrepancy between these results may be attributed to the antigens included in the two methods. Immunoblot strips contain nucleosomal antigens. Each nucleosome consists of DNA and histone proteins, and ELISA is loaded only with purified histone antigens. Obviously, the anti-nucleosomal antibodies direct to and react with not only histones, but also DNA, confirmed by the fact that in all serum samples, positive for anti-nucleosomal ANA, we have also found anti-dsDNA ANA.

For the rest of the ANA (anti-dsDNA and anti-SS-B) the difference between the two methods could not reach statistical significance which is similar to what has been found by other researchers.21,22

CONCLUSIONS
1. The comparative study of different immunological methods for initial ANA screening found good agreement between IFA-ANA on original serum-free cells McCoy-Plovdiv and ELISA. We recommend IFA for ANA screening (compared with ELISA) because of the richer collection of nuclear and other antigens and the capacity to detect low-titer ANA.
2. ELISA and immunoblot for specific ANA are the final stage in ANA diagnostics following a positive screening test. Despite the fact that in cases needing simultaneous detection of several specific ANA immunoblot is preferable compared to ELISA due to its faster and cheaper acquisition, we recommend ELISA for identification of ANA against extractable nuclear antigens because of its higher sensitivity.

ACKNOWLEDGEMENT
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СРАВНИТЕЛЬНОЕ ИССЛЕДОВАНИЕ ИММУНОЛОГИЧЕСКИХ МЕТОДОВ С ЦЕЛЬЮ ОПРЕДЕЛЕНИЯ СЫВОРОТОЧНЫХ АНТИНУКЛЕАРНЫХ АНТИТЕЛ

М. Мурджева, Н. Ряшева, М. Драганов, Л. Паунов

РЕЗЮМЕ

ВВЕДЕНИЕ: С целью определения сыровороточных антинуклеарных антител (ANA) применяются различные иммунологические методы. Стандартной техникой и „золотым“ стандартом для их доказательства является индиректный иммуннофлюоресцентный тест (IFA) с применением тканевых срезов или HEp-2 клеток. В качестве субстрата для IFA возможно и использование бессывороточной клеточной линии McCoy-Plovidiv. Экзисмовыявляющий иммуносуорбентный тест (ELISA) представляет другой метод для определения общих или специфических ANA. Иммуноблот также находит применение при доказывании специфических ANA.

МАТЕРИАЛ И МЕТОДЫ: В исследование включено 38 сывороток пациентов, скринированных на наличие общих ANA посредством ELISA (Trinity Biotech, NY, USA) и IFA-ANA и клеточной линии McCoy-Plovidiv. Позитивные пробы относительно специфичности доказанных ANA подтверждаются иммуноблотом (Orgentec Diagnostika, Germany) и ELISA.

РЕЗУЛЬТАТЫ: Между обозначены тестами определено несигнификантное различие (P > 0.05) и очень хорошее совпадение. Уверительные методы устанавливают очень хорошее совпадение при определении антител относительно SS-A, SS-B, dsDNA, умеренное относительно анти-Sm и анти-Sm/RNP и удовлетворительное относительно антигистоновых/нуклеозомных. Совпадение относительно анти-Scl-антител не доказано.

Заключение: IFA-ANA с бессывороточной клеточной линией McCoy Plovidiv и скрируирующая ELISA можно рекомендовать при определении общих ANA, а иммуноблот и ELISA при подтверждении и идентификации специфических ANA.