Screening for IgG Antinuclear Autoantibodies by HEp-2 Indirect Fluorescent Antibody Assays and the Need for Standardization

Susan S. Copple, MS, MT(ASCP)SI,1,2 S. Rashelle Giles, MT,1 Troy D. Jaskowski,1 Anna E. Gardiner,1 Andrew M. Wilson, MStat,1 and Harry R. Hill, MD1-4

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Abstract

We evaluated 5 commercially available HEp-2 antinuclear antibody (ANA) indirect fluorescent antibody (IFA) assays using patient serum samples from 45 patients with rheumatoid arthritis, 50 with systemic lupus erythematosus (SLE), 35 with scleroderma, 20 with Sjögren syndrome, 10 with polymyositis, and 100 healthy control subjects. In addition, 12 defined serum samples from the Centers for Disease Control and Prevention and 100 patient serum samples sent to ARUP Laboratories (Salt Lake City, UT) for ANA IFA testing were also examined (n = 372). Standardization among the HEp-2 IFA assays occurred when they exhibited the same titer ± 1 doubling dilution.

Agreement of the 5 assays was 78%. Within the specific groups of serum samples, agreement ranged from 44% in scleroderma serum samples to 93% in healthy control subjects, with 72% agreement in the SLE group. Variations in slide and substrate quality were also noted (ie, clarity, consistency of fluorescence, cell size, number and quality of mitotic cells).

Along with subjectivity of interpretation, HEp-2 IFA assays are also vulnerable to standardization issues similar to other methods for ANA screening. Patients with autoimmune disease often produce antinuclear antibodies (ANAs) that can be detected by indirect fluorescent antibody (IFA) techniques using a HEp-2 cell substrate. The ANA test should be ordered only when the patient’s history, symptoms, and physical examination findings are suggestive of a connective tissue disease (CTD). A negative ANA test is thought to rule out systemic lupus erythematosus (SLE); however, a positive result is not specific for CTD.1-6

The difficulty in diagnosing CTD dramatically increases the number of ANA tests ordered. At ARUP Laboratories, Salt Lake City, UT, more than 14,000 ANA tests are performed monthly and more than 160,000 per year. During the past decade, this increasing volume has led to the development of less labor-intensive methods, including enzyme linked immunoassay and multiplex microsphere immunofluorescent assays, for detecting ANAs or extractable nuclear antigen antibodies. These new methods do not agree 100% with IFA, and the lack of standardization and inconsistencies among these assays have frustrated many clinicians and laboratory professionals.7-11

In 2008, the American College of Rheumatology (ACR) initiated a task force to investigate and collect information from physicians to evaluate the extent of the problem. In August 2009, the ACR issued a statement declaring HEp-2 IFA as the preferred method for ANA screening.12 Using HEp-2 cells as the substrate, the IFA allows detection of more than 50 autoantibodies to 30 different nuclear and cytoplasmic antigens.9 These include antibodies to Golgi apparatus, mitochondria, Jo-1, ribonuclear protein, and others. Although it is ideal to report all fluorescence observed on the HEp-2 cells, many laboratories issue reports based on 5 or 6 basic IFA patterns, namely, homogeneous, speckled, nucleolar, centromere, peripheral/rim, and proliferating cell...
nuclear antigen, which are titered to end point. Each laboratory independently decides whether to indicate “cytoplasmic fluorescence observed,” spindle apparatus (NuMA [nuclear mitotic apparatus]), nuclear dots, or other fluorescent patterns.

It is well known that the IFA method is labor-intensive, subjective, and prone to reader bias. Interpretation requires readers to be well trained and experienced in performing the assay to maintain competence. Other variables affecting IFA assay results are the type of microscope bulb used, the hours in use, and the microscope.\textsuperscript{1,5,6,13-15} The need for standardizing of ANA testing continues to grow, as does controversy about the best test to use.\textsuperscript{7,9,16}

This study was designed to evaluate the diagnostic performance and standardization of 5 commercially available HEp-2 IFA assays used to screen for ANA. Serum samples from clinically defined disease samples, reference material, healthy subjects, and a group of samples sent from one clinical site to ARUP Laboratories for ANA IFA testing alone were examined. Based on the recommendation by the ACR to use HEp-2 IFA for screening ANA, we compared the agreement of 5 HEp-2 IFA assays read independently by 3 certified medical technologists to evaluate the standardization of ANA screening by indirect immunofluorescence.

**Materials and Methods**

**Clinical Samples**

We evaluated 372 serum samples that represented 50 clinically defined SLE cases and 45 clinically defined rheumatoid arthritis cases based on ACR criteria, 35 scleroderma cases, 20 cases of Sjögren syndrome, and 10 cases of polymyositis; 12 ANA human reference serum samples from the Centers for Disease Control and Prevention (CDC); samples from 100 healthy adult blood donors (70 women, 30 men; 19-59 years old); and 100 serum samples from 1 client sent to ARUP Laboratories for which ANA IFA testing alone was performed. All patient samples included in the study were deidentified according to the University of Utah Institutional Review Board–approved protocol No. 7275 to meet the Health Information Portability and Accountability Act patient confidentiality guidelines.

**Detection of ANAs by IFA**

HEp-2 ANA IFA assays from 5 manufacturers were evaluated: the Nova Lite HEp-2 ANA (INOVA Diagnostics, San Diego, CA), the Kallestad HEp-2 Cell Line Substrate (Bio-Rad Laboratories, Hercules, CA), the ANA/HEp-2 Cell Culture IFA Test System (Zeus Laboratories, Raritan, NJ), the HEp-2000 Fluorescent ANA-Ro Test System (Immuno Concepts, Sacramento, CA), and the ANA IFA: HEp-20-10 Test (Euroimmun, Boonton Township, NJ).

All assays were performed as stated in the manufacturer’s product insert. To analyze the serum samples at the same dilutions on all 5 ANA IFA assays, we used the starting dilution of 1:40 instead of a starting dilution of 1:100 as stated in the Euroimmun package insert. All samples were read on a Nikon Eclipse 400 microscope at ×200, independently by 3 board-certified medical technologists with 2 to 8 years’ experience reading IFA. The technologists were blinded to sample classification and each other’s readings. Owing to the unique cell line used for the Immuno Concepts HEp-2000 assay, the 3 medical technologists were trained in performing and reading the slides by representatives of Immuno Concepts.

**Results**

Results for all 372 samples were compared for agreement of the 3 technologists on each of the 5 HEp-2 IFA assays individually.\textsuperscript{Figure 1} Agreement was obtained when the 3 technologists’ results were within ± 1 doubling dilution of each other. As shown in\textsuperscript{Table 1}, there was excellent agreement (96%-99%) among readers; the Cronbach $\alpha$ ranged from 0.901 to 0.983.

The data were then separated by disease state, healthy blood donor, and unknown serum samples to examine how the 5 HEp-2 assays compared with each other in each unique subject group. The IFA results for the 3 readers were compared sample by sample in all 5 HEp-2 assays. The same criteria for agreement were used as stated earlier. Overall, the percentage of agreement for the 5 HEp-2 assays varied from
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Table 1
HEp-2 Antinuclear Antibody Indirect Fluorescent Antibody Assay Results Obtained for CDC Human Reference Serum Samples by Five Commercial Assays

<table>
<thead>
<tr>
<th>CDC Reference No.</th>
<th>Antibody</th>
<th>Kallestad</th>
<th>Immuno Concepts</th>
<th>Zeus</th>
<th>Euroimmun</th>
<th>INOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>dsDNA</td>
<td>1:80 H</td>
<td>1:80 M</td>
<td>1:160 H</td>
<td>1:80 H</td>
<td>1:80 H</td>
</tr>
<tr>
<td>2</td>
<td>SSB</td>
<td>1:80 S</td>
<td>1:320 S</td>
<td>1:1,280 S</td>
<td>1:320 S</td>
<td>1:320 S</td>
</tr>
<tr>
<td>3</td>
<td>U1-RNP, SSA, SSB</td>
<td>1:80 S</td>
<td>1:320 S</td>
<td>1:640 S</td>
<td>1:640 S</td>
<td>1:640 S</td>
</tr>
<tr>
<td>4</td>
<td>U1 RNP</td>
<td>1:160 S</td>
<td>1:640 S</td>
<td>1:640 S</td>
<td>1:640 S</td>
<td>1:640 S</td>
</tr>
<tr>
<td>5</td>
<td>Sm</td>
<td>1:80 S</td>
<td>1:640 S</td>
<td>1:320 S</td>
<td>1:320 S</td>
<td>1:320 S</td>
</tr>
<tr>
<td>6</td>
<td>Fibrillarin</td>
<td>1:80 N</td>
<td>1:80 N</td>
<td>1:160 N</td>
<td>1:80 N</td>
<td>1:160 N</td>
</tr>
<tr>
<td>7</td>
<td>SSA</td>
<td>&lt;1:40</td>
<td>&lt;1:40</td>
<td>1:40 S</td>
<td>&lt;1:40</td>
<td>1:40 S</td>
</tr>
<tr>
<td>8</td>
<td>Cant B</td>
<td>1:160 C</td>
<td>1:320 C</td>
<td>1:640 C</td>
<td>1:640 C</td>
<td>1:320 C</td>
</tr>
<tr>
<td>9</td>
<td>Scl-70</td>
<td>1:80 H</td>
<td>1:80 S</td>
<td>1:160 H</td>
<td>1:160 S</td>
<td>1:160 H</td>
</tr>
<tr>
<td>10</td>
<td>Jo-1*</td>
<td>&lt;1:40</td>
<td>&lt;1:40</td>
<td>&lt;1:40</td>
<td>&lt;1:40</td>
<td>&lt;1:40</td>
</tr>
<tr>
<td>11</td>
<td>PM/Scl-100</td>
<td>1:40 N</td>
<td>1:80 N</td>
<td>1:160 N</td>
<td>1:40 N</td>
<td>1:80 N</td>
</tr>
<tr>
<td>12</td>
<td>Ribo P*</td>
<td>&lt;1:40</td>
<td>&lt;1:40</td>
<td>&lt;1:40</td>
<td>&lt;1:40</td>
<td>&lt;1:40</td>
</tr>
</tbody>
</table>

C, centromere; H, homogeneous; M, mixed; N, nucleolar; S, speckled.
*Cytoplasmic antibodies.

44% in the scleroderma serum samples to 93% in the healthy blood donor group **Figure 2**.

Results for the CDC reference materials are shown in Table 1. All 5 assays detected anti-Jo-1 or anti-Ribo P as cytoplasmic fluorescence; however, the Immuno Concepts HEp2000 containing the transfected 60-kDa SSA/Ro, known for its ability to detect SSA, did not produce any fluorescent pattern for CDC reference material 7. The product information for CDC reference material 7 states “this serum reacts with cellular SS-A/Ro.”17

Titers and patterns produced by the 5 HEp-2 assays were compared for all 372 serum samples along with morphologic features and ease of reading. **Figure 3** and **Figure 4** show that the Kallestad assay produced lower titers in the SLE and scleroderma groups. This finding was consistent across all patient groups. Conversely, the Zeus and Euroimmun assays, containing a polyclonjugate, produced higher titers when compared with the other HEp-2 assays.

**Figure 2** Overall agreement between 5 HEp-2 indirect fluorescent antibody assays for each subject group of serum samples for antinuclear antibody measurement. Agreement was determined when all 3 technologists read the same titer ± 1 doubling dilution on all 5 HEp-2 assays. RA, rheumatoid arthritis; SLE, systemic lupus erythematosus.

**Figure 3** Eight systemic lupus erythematosus serum samples with discrepant titers on 5 HEp-2 assays. A unique symbol was assigned to each assay to demonstrate the actual titer produced for each patient serum sample.

**Figure 4** Eleven scleroderma serum samples with discrepant titers on 5 HEp-2 assays. A unique symbol was assigned to each assay to demonstrate the actual titer produced for each patient serum sample.
Inconsistency in the 5 HEp-2 assays in failing to produce equivalent titers when testing the same serum sample was most evident when comparing titers in disease categories, as demonstrated in Figures 3 and 4. For example, specimen 262 in the SLE group produced titers from 1:80 with Kallestad to 1:1,280 and 1:2,560 on Zeus and Euroimmun, respectively. Specimen 301 produced a titer of less than 1:40 on the Kallestad HEp-2 assay but was as high as 1:640 with the Inova assay.

Image II shows 1 SLE patient sample, at a 1:40 dilution, on each of the 5 HEp-2 assays. The striking differences in morphologic features, intensity of fluorescence, and percentage of mitotic cells can be seen.

Discussion

The difficulty in diagnosing CTD has led to an increased number of ANA tests being ordered, often to rule out the possibility of an autoimmune disease. During the past 2 decades, new methods for detecting and identifying ANAs have emerged to address the growing number of ANA tests requested. Along with these newer methods, there has been a growing number of HEp-2 ANA IFA assays that are commercially available. The growth of ANA testing, new methods, and increased number of manufacturers, unfortunately, have led to a lack of standardization among the assays.

Rheumatologists have expressed a growing concern about the lack of standardization for ANA testing. In August 2009, the ACR issued a statement recommending the use of HEp-2 IFA in all screening for ANA. Since the ACR recommendation, there have been numerous debates and many articles written about the use of the different methods for ANA IFA testing. Some argue that there is a large range of performance characteristics among laboratories using enzyme immunoassay; others argue there are often discordant results between laboratories running HEp-2 IFA, as can be seen in College of American Pathologists surveys. ANA titers often range from 1:40 to 1:1,280 between laboratories using HEp-2 IFA assays from the same vendor. Others have demonstrated that even though commercial enzyme immunoassay kits have been reported to have varying sensitivities and specificities, some have comparable and even higher diagnostic accuracy than the IFA method.

This study was designed to investigate the standardization of the HEp-2 IFA assays. Five HEp-2 IFA assays were evaluated by using 372 serum samples. All assays were run according to the manufacturer’s instructions. This study used the Euroimmun US Food and Drug Administration–cleared assay, which uses a 1:40 screening dilution (the European version uses 1:100). The technologist’s readings demonstrated excellent agreement among all serum samples when using an assay from the same vendor. When reading the results for the same serum samples on all 5 HEp-2 IFA assays, the agreement of the 5 assays was often poor, as demonstrated by 44% agreement with the scleroderma serum samples. The 50 serum samples from patients clinically diagnosed with SLE had an agreement of only 72% among the 5 different assays.

There are many factors that may contribute to the poor agreement between HEp-2 assays, such as the fluorescence to protein (F/P) ratio. The F/P ratio varied from 1.12 to 4.2 among the 5 assays.

The fixative used for the assay is a major source of inconsistency. Monce et al evaluated 2 fixatives on HEp-2 slides to determine which was better for detecting SSA/Ro. In that study, a pure acetone solution resulted in a 97.5% sensitivity and the alcohol/acetone combination resulted in an 81.3% sensitivity for the SSA antibody. In a study by Hahm and Anderer, 5 different fixatives for HEp-2 cell preparation were studied. The conclusion of this study was “The results demonstrate high variability in cell and nuclear morphology depending on the fixatives used.” This study indicated that the fixative may be the cause of the low 72% agreement among the 5 assays with the SLE serum samples.

The use of a polyconjugate vs an IgG-specific conjugate also resulted in variability among the assays. In December 1996, the National Committee for Clinical Laboratory Standards (NCCLS; now the Clinical and Laboratory Standards Institute) published “Quality Assurance for the Indirect Immunofluorescence Test for Autoantibodies to Nuclear Antigen (IF-ANA); Approved Guideline,” which offers a voluntary standard(s) developed by consensus of the clinical laboratory testing community. This guideline examines the use of an antihuman IgG-specific conjugate vs an antihuman polyconjugate (G, A, and M) or total IgG conjugate when testing for ANA by IFA on HEp-2 cells. It states that the use of a polyconjugate or IgG heavy/light chain conjugate will detect the IgM class antimuclear antibodies associated with rheumatoid arthritis, medications, and age, which are usually clinically insignificant. The organization favored the use of an antihuman IgG (Fc)-specific conjugate to enhance the positive predictive value of these ANA assays.

Other factors causing variability include growth time of the HEp-2 culture, pH of the assay reagents, and many others. The 3 technologists noted differences owing to the percentage of mitotic cells, the cell size, the use or overuse of Evans blue counterstain, the intensity of fluorescence, and the use of HEp-2 cells transfected with 60-kDa SSA/Ro, which caused difficulty in reading patterns. The requirement for interpreting HEp-2 IFA is an added problem with standardizing the IFA assay. The medical technologists who participated in this study had 2 to 8 years of experience reading and interpreting 70 to 300 ANA IFA samples daily.
From the results obtained in this study, we conclude that HEP-2 IFA assays are vulnerable to standardization problems similar to other methods for ANA screening, and additional problems are due to subjectivity of interpretation. It is recommended that the laboratory personnel inform physicians of the method used for ANA testing and make them aware of the problems inherent in the IFA assay.21,22

References