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Recent Approaches To Optimize Laboratory Assessment of Antinuclear Antibodies

Anne E. Tebo

Department of Pathology, University of Utah School of Medicine and ARUP Laboratories, Salt Lake City, Utah, USA

ABSTRACT The presence of antinuclear antibodies (ANAs) is a hallmark of a number of systemic autoimmune rheumatic diseases, and testing is usually performed as part of the initial diagnostic workup when suspicion of an underlying autoimmune disorder is high. The indirect immunofluorescence antibody (IFA) technique is the preferred method for detecting ANAs, as it demonstrates binding to specific intracellular structures within the cells, resulting in a number of staining patterns that are usually categorized based on the cellular components recognized and the degree of binding, as reflected by the fluorescence intensity or titer. As a screening tool, the ANA patterns can guide confirmatory testing useful in elucidating a specific clinical diagnosis or prognosis. However, routine use of ANA IFA testing as a global screening test is hampered by its labor-intensiveness, subjectivity, and limited diagnostic specificity, among other factors. This review focuses on current efforts to standardize the nomenclature of ANA patterns and on alternative methods for ANA determination, as well as on recent advances in image-based computer algorithms to automate IFA testing in clinical laboratories.

KEYWORDS antinuclear antibodies, autoimmunity, diagnosis, methodologies

Address correspondence to anne.tebo@hsc.utah.edu.

The detection of autoantibodies against intracellular antigens, called antinuclear antibodies (ANAs), is important in the diagnosis of systemic autoimmune rheumatic diseases (SARDs) such as systemic lupus erythematosus (SLE), Sjögren's syndrome (SjS), mixed connective tissue disease (MCTD), systemic sclerosis (SSc), and idiopathic inflammatory myopathies (IIMs). Therefore, testing for ANAs is a logical first step in the differential evaluation of patients when a systemic autoimmune etiology is suspected. Timely diagnosis of SARDs is challenging due to the wide spectrum of overlapping symptoms. Furthermore, while the frequency of ANAs is highest in patients with SARDs, these antibodies are also found in patients with organ-specific autoimmune diseases (e.g., autoimmune liver diseases and Hashimoto's thyroiditis), certain infections, cancer, and advanced age and in some healthy individuals (1–3). Thus, ANA testing in people with a low pretest probability for a SARD can cause undue concern (1, 4–6).

The use of ANA detection as a diagnostic test dates back to the original observation of the "lupus erythematosus (LE) cell'" by Hargraves and colleagues in 1948 (7). Until then, a diagnosis of SLE often could not be established unless tissue specimens were obtained from the affected patient. Early studies to detect ANAs with an indirect immunofluorescence antibody (IFA) technique used kidney or liver sections from rats or mice as substrates (8–10). The use of HEp-2 cells, of human larynx epithelioma origin, provided increased sensitivities for detecting ANAs, probably due to their ability to divide rapidly *in vitro* as well as the presence of large nuclei, which allowed optimal detection of patterns associated with specific autoantibodies in SARDs (4, 9, 10). Although the ANA IFA method continued to be plagued by a number of analytical and diagnostic challenges, increasing recognition of the use of autoantibodies to diagnose

TABLE 1 Commonly reported antinuclear antibody patterns, related autoantigens, and clinical associations[a]

Pattern	Common autoantibody targets[b]	Clinical associations[c]
Homogeneous	dsDNA, histones, chromatin/nucleosomes	SLE, drug-induced SLE
Speckled	U1-RNP, SSA/Ro, SSB/La, Smith, topo I (Scl-70), Mi-2	SLE, SjS, SSc, IIMs, MCTD
Centromere	Centromeric proteins	SSc, PBC
Nucleolar	PM/Scl, RNA polymerase, U3-RNP, Th/To	SSc, IIMs, Raynaud's syndrome overlap
Cytoplasmic	Ribo P, Jo-1, other tRNA synthetases, SRP	SLE, IIMs, PBC, interstitial lung disease

[a]The table shows the commonly recognized ANA patterns detected using the HEp-2 substrate and their associated antibody markers, which are routinely tested in clinical laboratories. The homogeneous, speckled, centromere, and nucleolar patterns represent "true" ANAs and are associated with nuclear staining.
[b]dsDNA, double-stranded DNA; RNP, ribonucleoprotein; topo I, topoisomerase I; Mi-2, chromodomain helicase DNA-binding protein 4; PM, polymyositis; Scl, scleroderma; Th/To, Th/To ribonucleoprotein; Ribo P, ribosomal P protein; SRP, signal recognition particle.
[c]SLE, systemic lupus erythematosus; SjS, Sjögren's syndrome; SSc, systemic sclerosis; IIMs, idiopathic inflammatory myopathies; MCTD, mixed connective tissue disease; PBC, primary biliary cholangitis.

and to further stratify SARDs, as well as efforts to harmonize the nomenclatures for testing and reporting, makes this a powerful screening tool (1, 11–17). Thus, testing for ANAs or ANA-specific autoantibodies has been incorporated in a few guidelines on diagnostic criteria. For example, a positive ANA finding was incorporated as a diagnostic criterion for SLE by the American College of Rheumatology (ACR) in the early 1980s, with subsequent revision in 1997 (18, 19). In 2013, a joint committee of the ACR and the European League Against Rheumatism (EULAR) formulated a new set of SSc classification criteria, which included ANA-specific autoantibodies, namely, antitopoisomerase I (anti-topo I) (or anti-Scl-70), anticentromere (ACA), and anti-RNA polymerase III (anti-RNAP III) (20).

INDICATIONS FOR ANTINUCLEAR ANTIBODY TESTING

ANA testing is commonly used to assess the likelihood of a SARD diagnosis, with relevant information coming from the identification of antibodies bound to specific intracellular targets. While the immunological mechanisms underlying these interactions are poorly understood, the presence of ANAs generally represents abnormal responses to self-antigens, a key feature of autoimmunity. In routine clinical laboratory evaluations, ANAs are generally categorized based on the recognition of homogeneous, speckled, centromere, and nucleolar patterns (1, 2, 15–17). While ANAs are used as part of diagnoses for some SARDs (e.g., SLE, MCTD, and SjS), their presence may serve as an important diagnostic support for others (SSc, IIMs, secondary SjS, and juvenile arthritis).

The recognition of a well-defined ANA staining pattern using the HEp-2 cell substrate may be helpful in determining the most likely autoantibodies present, as well as suggesting possible clinical associations for known specificities. In this regard, a positive ANA screening pattern can guide confirmatory testing and may also be useful for elucidating a specific clinical diagnosis or prognosis. Table 1 shows some of the more common ANA patterns, their associated autoantibody specificities, and their clinical correlations. Antibodies to double-stranded DNA (dsDNA) and Smith (Sm) are commonly associated with SLE, while those targeting SSA-52 and Ro60 are important markers of SjS, although they are seen in a variety of other SARDs (11). Antibodies to ribonucleoproteins (RNPs) or small nuclear RNP (snRNP) complexes may be seen in patients with SLE or MCTD. Patients with MCTD have features of different rheumatic diseases, and the presence of anti-U1-RNP antibodies is considered important for their diagnosis. ANA tests are also positive in almost all cases of SSc, with certain patterns associated with multiple antigenic targets being crucial for clinical stratification and management (1, 14). For example, the ANAs in SSc (anti-RNAP III, anti-Th/To, anti-polymyositis (PM)/scleroderma (Scl), anti-Ku, and anti-U3-RNP) target proteins that are found in the nucleus and nucleolus. These antibodies are associated with certain manifestations of SSc, including organ involvement, and survival prognosis. Of the many SSc antibodies, only three main specificities (ACA, anti-topo I, and anti-RNAP III antibodies) are recommended in the classification criteria (20). Antibodies to Th/To and U3-RNP are also considered specific for SSc, while autoantibodies to PM/Scl, U1-RNP,

and Ku are usually detected in patients with overlap syndromes, such as PM and SSc or SLE and SSc (14). In recent years, testing for specific ANAs has become useful in the evaluation of IIMs (12, 13). While the patterns and specificities of ANAs in IIMs are quite diverse (1, 3, 15), identification of certain autoantibodies is useful both for diagnosing and distinguishing between subtypes of myositis and for predicting or monitoring the development of additional clinical manifestations, including organ involvement, and risks for cancers (12, 13). In general, these autoantibodies are categorized as myositis-specific antibodies (MSAs) or myositis-associated antibodies (MAAs). MSAs are unique, generally mutually exclusive, and diagnostic for IMMs, while MAAs do occur in variety of SARDs or connective tissue diseases. These antibodies generally target complex antigens in their native configuration and require specialized forms of testing that are not available in most clinical laboratories.

CONTEMPORARY ISSUES ON ANTINUCLEAR ANTIBODY NOMENCLATURE

Detection of ANAs by the IFA technique demonstrates binding to specific intracellular structures within the cells, resulting in a number of staining patterns that are usually categorized based on the subcomponents recognized and the degree of binding reflected by the fluorescence intensity or titer. The provision of the ANA pattern and titer is considered to be of added clinical value, especially with respect to other methods for ANA detection (1–3, 15, 21–29). As mentioned above, the patterns most commonly recognized and reported by clinical laboratories are those staining the nuclear region, referred to as homogeneous, speckled, centromere, and nucleolar (1, 2, 15–17). With the use of HEp-2 cells as the substrate, there is increasing awareness that additional nuclear staining patterns, as well as reactivity with cell compartments outside the nucleus (cytoplasmic patterns) and features associated with different stages of mitosis (mitotic patterns), may be of diagnostic relevance in SARDs (1–3, 15–17). However, the presence or amount of some autoantigens associated with these patterns can vary among substrates from different manufacturers (30). Furthermore, the expertise required to recognize and to distinguish the different patterns and their variants may not be available in most clinical laboratories. Thus, which additional patterns are reported differs among laboratories, as elegantly outlined in the recent international recommendations for the assessment of ANAs (1) and as described below.

Recently, recommendations for assessing ANAs (1) and standardized nomenclature from the International Consensus on ANA Patterns (ICAP) have been published (15–17). The experts for ICAP recommended that patterns be categorized in three major groups, namely, nuclear, cytoplasmic, and mitotic patterns (www.ANApatterns.org) (15). The ICAP committee also described 28 distinct patterns on the HEp-2 substrate and provided each pattern an anticell (AC) number of 1 through 28, with an assignment of competent or expert level (15). Of the 28 patterns, the homogeneous, speckled, centromere, discrete nuclear dots, nucleolar, and dense fine speckled nuclear patterns (AC-1 to AC-14) are considered true ANA patterns and belong to the competent level. For the patterns associated with cytoplasmic staining (AC-15 to AC-23), the ICAP classification tree indicates the fibrillar, speckled, reticular/antimitochondrial antibody (AMA), and polar/Golgi-like patterns, as well as rods and rings, as belonging to the competent level. All of the patterns associated with the mitotic category were assigned expert level recognition. Of the patterns associated with competent level proficiency, discrete nuclear dots are less commonly recognized (1), and studies on the dense fine speckled pattern do not currently indicate technical competencies (3, 15–17). Nevertheless, the main goals of the ICAP are to promote harmonization of autoantibody nomenclature and interpretation and to optimize the usage of ANA findings in patient care (16). Along these lines, a key feature of the ICAP nomenclature is to provide a definition and description of each of the main ANA patterns, as well as their associations with specific autoantibodies and their clinical correlations. Despite the recognition that nonnuclear patterns (cytoplasmic and mitotic patterns) are clinically relevant, no consensus on reporting these as ANA negative or positive was reached. Central to this

contention were the implications for existing diagnostic/classification criteria for ANA-associated diseases such as autoimmune hepatitis (16, 31).

ASSESSMENT OF ANTINUCLEAR ANTIBODIES

Since the adoption of ANA assessment as a routine clinical laboratory test, the techniques for ANA detection and/or measurement have evolved and now encompass a variety of immunological methods (21–30). The IFA method using HEp-2 cells is considered the standard method for detecting ANAs, with a number of pros and cons (1–4, 21, 22, 24–30). Central to the analytical challenges associated with ANA IFA testing are its labor-intensiveness (with significant amounts of training being required for competence), subjectivity in titer and pattern recognition, poor standardization of reagents, and a declining workforce in clinical laboratories (1–4, 15, 30, 32). Thus, in some laboratories, the ANA IFA technique has been replaced by high-throughput, less subjective methods, including enzyme-linked immunosorbent assays (ELISAs) and multiplex assays such as line immunoassays (LIAs) and multiplexed bead assays (MBAs) (21–29). The non-IFA assays differ in the source, purity, concentration, and binding capacity of the antigens, the reference materials or standards used in assay development, the secondary antibodies (conjugates), and the detection signals. Therefore, it is not uncommon to observe differences in diagnostic accuracies between immunoassays, even among those of equivalent analytical principles, for patients in the same cohort (21–24). Concerns regarding decreased sensitivities of the newer high-throughput assays and clinician awareness of the different methodologies in diagnostic laboratories and their associated limitations led both the ACR (33) and an international group of experts (1, 15–17) to develop recommendations for ANA nomenclature, assessment, and interpretation. Despite the recognized limitations of ANA IFA testing using HEp-2 cells as the substrate, the method remains the acceptable reference method for ANA detection, based on its higher sensitivity due to inclusion of the largest number of antigens in their native conformations.

INDIRECT IMMUNOFLUORESCENCE ANTIBODY TECHNIQUE

The IFA technique is based on a simple immunological principle that involves incubation of patient sera with substrate (cell line or tissue section) fixed to a glass slide. At present, the HEp-2 cell line is the preferred substrate for the detection and quantification of ANAs, because the cell nuclei are large and express a wide variety of antigens associated with SARDs. Serial dilutions of positive samples are tested to obtain an endpoint titer. Screening dilutions vary between laboratories, which should be considered when interpreting results. At a serum dilution of 1:40, a significant number of healthy individuals may test positive for ANAs; however, the prevalence decreases when the screening dilution is increased, with associated losses in sensitivities for certain SARDs (4). Thus, the performance characteristics of ANA IFA tests are recognized to be variable. With the exclusion of nonstandardized nomenclature, the cellular concentrations of antigens, conditions of cell fixation, microscope quality and type, specificity of the anti-IgG reagents, and cutoff determinations are factors likely to contribute to inconsistencies in the performance characteristics of ANA IFA tests (1, 3, 15–17, 30). The amounts of certain autoantigens in HEp-2 cells, such as SSA-A/Ro60, can limit the sensitivity for the detection of antibodies directed against this molecule in affected patients (30). A modified HEp-2 cell line transfected with human 60-kDa Ro/SSA has been reported to have the additional advantage, compared with the regular HEp-2 substrate, of detecting anti-Ro antibodies in ANA-negative sera (30, 34, 35). Other attributes of the ANA kit, such as cell fixation procedures and anti-IgG reagents, remain nonstandardized, and various recommendations and ongoing efforts to achieve harmonization and standardization have been published (1, 2, 36). The appropriate cutoff value for positivity remains one of the most contentious issues in ANA testing in clinical practice (1–6). Using a screening dilution of 1:80, the ANA prevalence in a U.S. population ≥12 years of age was reported to be 13.8% (95% confidence interval [CI], 12.2% to 15.5%) (5). The ANA prevalence observed in the U.S. study was comparable to

that observed in a recently published report from Germany (5). As in other previous reports, women had higher frequencies of ANA positivity than did men in both studies (5, 6, 37), which could be related to reproductive and hormonal factors (38). While increasing frequency of ANA positivity with increasing age and according to gender has been reported, there are likely other factors involved. Another dilemma associated with positive ANA results in otherwise healthy individuals has to do with the concept of preclinical disease onset. Unlike in infectious disease serological testing, where a positive antibody result may denote active or passive exposure and false-positive results can easily be determined, it is now recognized that autoantibodies may precede clinical disease onset (39). In this regard, ANA positivity could suggest a predisposition for autoimmunity, with ANA expression preceding overt clinical disease, as described for a number of autoimmune conditions (40). On the other hand, false-positive results for apparently healthy individuals or non-SARD patients may be related to specific binding and/or cross-reactivity with undefined or defined targets such as antibodies to DFS70 (41). The DFS70 pattern is associated with autoantibodies binding to a ubiquitously expressed protein called lens epithelium-derived growth factor (LEDGF) (or p75 or *psip1* gene product), which was first described by Ochs and colleagues in 1994 (42) and was subsequently reported for patients with atopic dermatitis and asthma (43). Although antibodies to DFS70 have been associated with a variety of clinical conditions, the antibodies commonly occur in healthy individuals. Importantly, low levels of expression of these antibodies might occur in patients with autoimmune diseases characterized by ANA production (44). Therefore, the presence of anti-DFS70 antibodies might exclude the diagnosis of an autoimmune disease, although the possible association with other conditions affects the manner in which positive test results are interpreted in the context of patient evaluations. Using HEp-2 cells, the DFS70 pattern is characterized by dense heterogeneous fine speckled staining of the nucleoplasm in interphase and speckled staining tightly associated with chromatin during mitosis (15, 16). However, this pattern is not easily discernible even by well-trained individuals and may be reported as a speckled pattern (3, 15–17). The availability of immunoassays targeting the DFS70 antigen is likely to help minimize the subjectivity associated with interpreting this pattern in IFA testing.

ALTERNATIVE IMMUNOASSAYS FOR DETECTING ANTINUCLEAR ANTIBODIES

Increasing requests and an associated surge in test volume for ANA testing in clinical laboratories have led to the need for high-throughput, less labor-intensive, more objective methods for detecting and measuring ANAs. A variety of manual or automated single or multiplex immunoassays (examples of the most commonly used methods include ELISAs, chemiluminescence immunoassays [CLIAs], LIAs, and MBAs), with different performance characteristics, have been developed and reported in the literature (21–30). The advantages of these alternative approaches to ANA IFA testing include the ease of use, the possibility of performing quantitative high-throughput analyses, the lack of subjectivity, and the consolidation of ANA-related tests in a single platform. The main disadvantages of the use of non-IFA methods for detection of ANAs are related to the unreliability of negative results, due to the restricted number of antigens, and the diagnostic dilemma when the screening results are positive and subsequent ANA IFA testing results are negative.

Of all the non-IFA methods, ELISA is one of the most common and versatile techniques available in clinical laboratories. Commercially available ANA ELISAs have various configurations of antigens (substrates) and secondary antibodies (23, 24, 28, 30). While most ELISAs for the detection of ANAs use HEp-2 cell extracts spiked with a combination of purified, recombinant, or native proteins associated with common SARDs, quite a few do not include the HEp-2 cell extract. Thus, depending on the formulations of the ELISA, the clinical performances vary based on the specific SARD under evaluation. For ANA ELISAs designed with a complex mixture of antigens such as the HEp-2 cell extract with unknown concentrations, antigens can compete for binding

to the available surface, affecting the presentation of some antigenic targets. Alternatively, conformational changes in antigenic structures may result in inaccurate results. In general, ANA ELISAs based on the use of defined antigenic targets have lower sensitivities and excellent specificities for connective tissue diseases and may not be useful in the evaluation of autoimmune liver diseases or SARDs that target the complex autoantigens seen in IMMs and SSc (1, 3, 13, 14, 23, 45). The analytical principles for CLIAs are different from those for ELISAs; however, the concept of their use to detect ANAs is similar to that for ELISAs (29). Unlike ELISAs, the enzymes used in CLIAs convert a substrate to a reaction product that emits a photon of light, instead of developing a visible color. CLIAs have a wider analytical measurement range and are more sensitive and easier to automate than ELISAs, making them preferred assays for high-throughput analysis.

Multiplex assays are also similar in principle to ELISAs and CLIAs but with the capacity to detect multiple independent antibodies associated with ANAs. In LIAs or MBAs, a selection of antigens, usually purified, native, or recombinant proteins or synthetic peptides, are bound in parallel lines to a nylon membrane or beads in order to provide a substrate for antibody detection (25, 27, 29). Following exposure to diluted patient specimens, the presence of autoantibodies is detected with an anti-human IgG reagent conjugated to an enzyme or fluorophore. This system can be automated and has the advantage of providing the simultaneous detection of several autoantibodies with diagnostic relevance. MBAs are technologically more sophisticated than LIAs, with the capacity to detect significantly more autoantibodies. MBAs utilize a series of beads of distinct immunofluorescence intensities, each coated with a different antigen. Autoantibodies to different antigens can be determined either quantitatively or qualitatively, by determining reactivity to the different beads using fluorescence or chemiluminescence readouts (25, 27, 29). In the case of LIAs, the principle of antibody detection is similar to that of ELISAs, where the secondary antibody is conjugated to an enzyme and the presence of autoantibodies is determined qualitatively or quantitatively by densitometric analysis.

Compared to the IFA method, alternative methods to detect ANAs have lower sensitivities or poor predictive values, even with the use of the HEp-2 cell extract (2, 3, 21, 23, 24, 45, 46). This is particularly important in SARDs such as SSc and IMMs (3, 13, 21, 46, 47). For example, patients with myositis have been reported to test positive for a variety of antibodies that target complex autoantigens (for example, tRNA synthetases, signal recognition particle [SRP], or Mi-2) associated with specific ANA IFA patterns (15), which currently require more specialized forms of testing, such as protein immunoprecipitation or RNA immunoprecipitation techniques, that are not readily available in clinical diagnostic laboratories. Although a number of these analytes are available for testing using alternative ANA immunoassays, their diagnostic accuracies are limited, mainly due to the complexity of the autoantibody targets (46, 47). The main advantages of using alternative methods are better specificities, faster turnaround times, objectivity, high throughput, and arbitrary values that may be useful for predicting more reliably the more common SARDs, such as SLE, SjS, and MCTD, based on the use of more commonly defined autoantibody targets. Compared to ELISAs, the multiplex assays have the potential to be informative tools for autoantibody cluster analysis or evaluation of diseases with multiple autoantibody specificities, such as SSc, SLE, and IMMs.

AUTOMATION OF ANTINUCLEAR ANTIBODY IMMUNOFLUORESCENCE ANTIBODY TESTING

Despite the known challenges associated with ANA IFA testing, a major strength of this technique as a screening tool for SARDs lies in the diversity of staining patterns and the insights the patterns provide for specific autoantibodies and their clinical associations, as well as being a versatile tool for novel biomarker discovery. Significant efforts have been made to minimize labor-intensiveness and subjectivity in interpreting ANA patterns through the development and validation of front-end processors for the

TABLE 2 Characteristics of automated readers for detecting antinuclear antibodies by IFA testing[a]

Automated system	No. of samples/h	Full automation	No. of patterns recognized[b]	Nuclear stain[c]
Aklides	48–60	No	6[d]	DAPI
EUROPattern	90	Yes	7[d]	PI
Helios	150	Yes	7[d]	None
Image Navigator	90	Yes[e]	Positive/negative	None
NOVA View	48–60	Yes[e]	5[d]	DAPI
Zenit G-Sight	14–48	No	5[d]	None
Cytospot	96	Yes	Positive/negative	None

[a]Adapted from Tozzoli et al. (49) and Bizzaro et al. (50), with modification.
[b]Aklides: cytoplasmic, homogeneous, speckled, nucleolar, centromere, and multiple nuclear dots patterns; Helios: centromere, cytoplasmic, homogeneous, nuclear membrane, nuclear dots, nucleolar, and speckled (granular) patterns; EUROPattern: homogeneous, speckled, nucleolar, centromeric, nuclear dots, nuclear membrane, and cytoplasmic patterns; NOVA View: homogeneous, speckled, centromere, nucleolar, and nuclear dots cytoplasmic patterns; Zenit G-Sight: homogeneous, nucleolar, speckled, centromere, and mitochondrial patterns.
[c]DAPI, 4′, 6-diamidino-2-phenylindole; PI, propidium iodide.
[d]Titer prediction capabilities.
[e]Connection to pipetting station.

handling and washing of slides and automated software for image recognition (21, 29, 48, 49). With the automated computer-assisted pattern recognition systems, the main operating principles include acquiring, storing, and analyzing digital images of stained HEp-2 cell substrates and displaying them on high-resolution monitors for review (48). Automation of both ends of ANA testing (front-end processing and digital reading) significantly minimizes the manual and analytical challenges of ANA IFA testing. There are a number of commercially available systems for automated reading of ANA IFA results (Table 2) based on the principles outlined above, with some variations (13, 49–58). These systems differ from each other with respect to the use of DNA-binding counterstains (4′,6-diamidino-2-phenylindole [DAPI], propidium iodide, or none), the cell substrate used (HEp-2 versus HEp-2000, with restriction to the manufacturer's slides), throughput (samples per hour), the number of patterns that can be identified, titer prediction, front-end automation (slide processors), and other software features (49, 50). Some of the automated ANA IFA readers also contain pattern image libraries, which can serve as powerful tools for training end-users, as well as providing comparisons to aid in assigning patterns.

Preliminary studies based on the use of single automated ANA IFA systems or a combination of systems indicate comparable performance between manual and automated ANA IFA readings for negative and positive samples, with some variations in detecting some basic patterns and reporting results for samples with more than one ANA IFA pattern (50–58). Bizzaro and colleagues (50) were the first to compare the diagnostic accuracies of six commercial systems for automated ANA IFA readings with the same series of sera. Although the overall performance for positive or negative results was acceptable, the accuracy of pattern recognition, restricted to the most common patterns reported (homogeneous, speckled, nucleolar, centromere, multiple nuclear dots, and cytoplasmic patterns), was limited, ranging from 52% to 79%, with significant challenges in interpreting samples with more than one pattern (mixed patterns). Those findings appear to be consistent with others that report misinterpretation difficulties for the common and mixed patterns, in addition to false-negative results for cytoplasmic, nuclear dots, and nuclear membrane patterns (50–58). For the available ANA IFA automated readers, the final result is provided by the operator. Thus, while the automated readers may show acceptable correlations for positive and negative results, the inherent subjectivity in ANA interpretation, with its clinical implications, does persist. This is particularly important because a consensus regarding ANA nomenclature remains poorly defined (1, 3, 15–17). Correct determination of ANA patterns is important for confirming a number of autoantibody specificities and for timely diagnosis, to minimize severe outcomes. While ANA IFA automated readers have

been demonstrated to improve intra- and interlaboratory correlations (59, 60), it is likely that factors such as the HEp-2 substrate and secondary antibodies may also significantly affect the appearance of certain ANA specificities. In addition, the different systems for acquiring and analyzing ANA HEp-2 images are likely based on different programming parameters (61).

PERSPECTIVES ON FUTURE DIRECTIONS

Recent advances in image-based computer algorithms have significantly affected how ANAs are detected and measured in clinical laboratories. Initial studies showing that automated IFA readers are generally capable of categorizing results as positive or negative are quite encouraging. However, the recognition that these readers show limited correlations for certain patterns raises concerns regarding subjectivity, as interpretations are still dependent on the technical operators. While it is likely that these outcomes may be due to a lack of consensus in identifying and categorizing patterns, the inability of current software programs to classify images correctly cannot be completely ruled out. Given that only a few of the patterns are currently reported, it is likely that efforts to make the automated readers more robust are needed. Incorporation of recent guidance on ANA classification and nomenclature, as well as definition of acceptable reagents (including the quality of HEp-2 cell substrates), and standardization of techniques to capture and to classify images with next-generation algorithms may lead to better interassay correlations. In the meantime, laboratories should focus on training personnel and building image libraries using guidance for assessing ANAs, for improved inter- and intralaboratory correlations and clinical classification of patients. Workshops designed to train clinical laboratory scientists, revised software programs to accurately determine ANA patterns, and establishment of proficiency programs would also be helpful in optimizing the detection and measurement of ANAs.

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Anne E. Tebo is Associate Professor in the Department of Pathology at the University of Utah School of Medicine and Medical Director of the Autoimmune Immunology Laboratory at ARUP Laboratories in Salt Lake City, Utah. She received her doctoral degree from the Eberhard Karls Universität (Tübingen, Germany) and completed postdoctoral training in immunology in the Department of Microbiology at the University of Alabama at Birmingham. Dr. Tebo continued her training with a clinical immunology fellowship in the Department of Pathology at the University of Utah School of Medicine and is board certified in medical laboratory immunology by the American Board of Medical Laboratory Immunology (ABMLI). Dr. Tebo leads the development and evaluation of biomarkers for autoimmune diseases, which includes efforts to harmonize and to standardize serological tests commonly used for disease assessment.