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Antinuclear Antibodies Testing Method Variability: A Survey of Participants in the College of American Pathologists' Proficiency Testing Program

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ABSTRACT. ***Objective.*** This study was conducted to determine the spectrum of laboratory practices in antinuclear antibody (ANA) test target, performance, and result reporting.

Methods. A questionnaire on ANA testing was distributed by the Diagnostic Immunology and Flow Cytometry Committee of the College of American Pathologists (CAP) to laboratories participating in the 2016 CAP ANA proficiency survey.

Results. Of 5847 survey kits distributed, 1206 (21%) responded. ANA screening method varied: 55% indirect immunofluorescence assay, 21% ELISA, 12% multibead immunoassay, and 18% other methods. The name of the test indicated the method used in only 32% of laboratories; only 39% stated the method used on the report. Of 644 laboratories, 80% used HEp-2 cell substrate, 18% HEp-2000 (HEp-2 cell line engineered to overexpress SSA antigen, Ro60), and 2% other. Slides were prepared manually (67%) or on an automated platform (33%) and examined by direct microscopy (84%) or images captured by an automated platform (16%). Only 50% reported a positive result at the customary 1:40 dilution. Titer was reported to endpoint routinely by 43%, only upon request by 23%, or never by 35%. Of the laboratories, 8% did not report dual patterns. Of those reporting multiple patterns, 23% did not report a titer with each pattern.

Conclusion. ANA methodology and practice, and test naming and reporting varies significantly between laboratories. Lack of uniformity in testing and reporting practice and lack of transparency in communicating the testing method may misdirect clinicians in their management of patients.

Key Indexing Terms: antinuclear antibody test, diagnostic errors, diagnostic reagent kits, ELISA, IFA, systemic lupus erythematosus

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Antinuclear antibodies (ANA) are an important diagnostic indicator of several autoimmune diseases, including systemic lupus erythematosus (SLE), systemic sclerosis (SSc), and mixed connective tissue disease (MCTD)[1]. ANA testing often serves as an initial gateway test in the evaluation of patients with symptoms suggestive of autoimmunity[2]. Multiple test designs have been employed to detect ANA, including indirect immunofluorescence assay (IFA), ELISA, and multibead immunoassays. The American College of Rheumatology (ACR) recommends IFA using HEp-2 as the gold standard for ANA testing. The ACR also recommends that clinical laboratories that do not use an ANA IFA specify their assay method when reporting results[3,4]. To determine the adherence to this guideline, we conducted a survey of ANA testing methods in clinical laboratories to determine the extent in which the ACR position statement was adopted. The survey also examined the variability in the testing and reporting practices of the laboratories using the IFA methodology.

MATERIAL AND METHODS

In April 2016, 5847 survey kits (Survey S-A 2016) were distributed to clinical

laboratories that participated in the College of American Pathologists' (CAP) Proficiency Testing Program; these laboratories were in the United States and in other countries. A questionnaire was included in the proficiency survey covering topics including ANA testing methods, cells used, slide preparation methods, image capture and interpretation, and reporting of results. The number of responders for each topic varied because information provided in response to the questionnaire was in some cases incomplete or insufficiently detailed.

The Clinical Laboratory Improvement Act (CLIA) mandates laboratory participation in a proficiency testing program. CAP offers a proficiency testing program in which commercially sourced and vetted blinded samples are provided to laboratories and testing results are analyzed. As participants in the CAP proficiency program, laboratories agree to the CAP use of survey results in a deidentified fashion. Institutional review board review is not required.

RESULTS

Response rate and location. A total of 1206 (21%) clinical laboratories responded to the survey (942 from the USA and 264 from other countries). Survey questions are enumerated in the Supplementary Material (available with the online version of this article).

Testing methods. Of the 1206 responding laboratories, 55% used ANA IFA, 21% used ELISA, 12% used a multibead immunoassay, and 18% used other methods (Figure 1A). The summed total for several answers is > 100% of the total respondents because some laboratories used more than 1 method. In the United States, 50% of laboratories used IFA versus 75% in international laboratories. Immunobeads were used for ANA screening by 14% of US respondents versus 4% of international respondents. Of the 1015 laboratories that reported using a laboratory-specific generic method if a specific method was not requested, 56% used IFA, 23% used ELISA, 11% used a multibead immunoassay, and 10% used other methods (Figure 1B). In the US, if a specific method was not requested, 52% of reporting laboratories used IFA, 23% ELISA, 14% immunobeads, and 12% used other methods. This contrasts with international laboratories, in which 71% used IFA, 21% ELISA, 3% immunobeads, and 6% other methods.

Naming the method used. Of the 1015 laboratories that provided a test name, the test name reflected the method used in only 328 (32%). Of 1009 laboratories that responded to the methods question, only 396 (39%) indicated on the laboratory report which ANA testing method was used.

Cell type. Of the 644 laboratories responding to the cell type question, 80% used HEp-2 substrate, 18% used HEp-2000, and 2% used other (Figure 2). HEp-2000 cell substrate was used in only 3% of international respondent laboratories versus 24% in the US.

Slide preparation. Of the 646 laboratories responding to the slide preparation method question, 435 (67%) prepared slides manually and 211 (33%) used an automated platform.

Image capture and interpretation. Of the 251 responding laboratories, 210 (84%) examined immunofluorescence images manually and 41 (16%) captured them by automated platform. Automated platform image interpretation was performed by 1 laboratory out of 636 (< 1%), while 607 (95%) laboratories used personnel to interpret images and 28 (4%) used both personnel and automation.

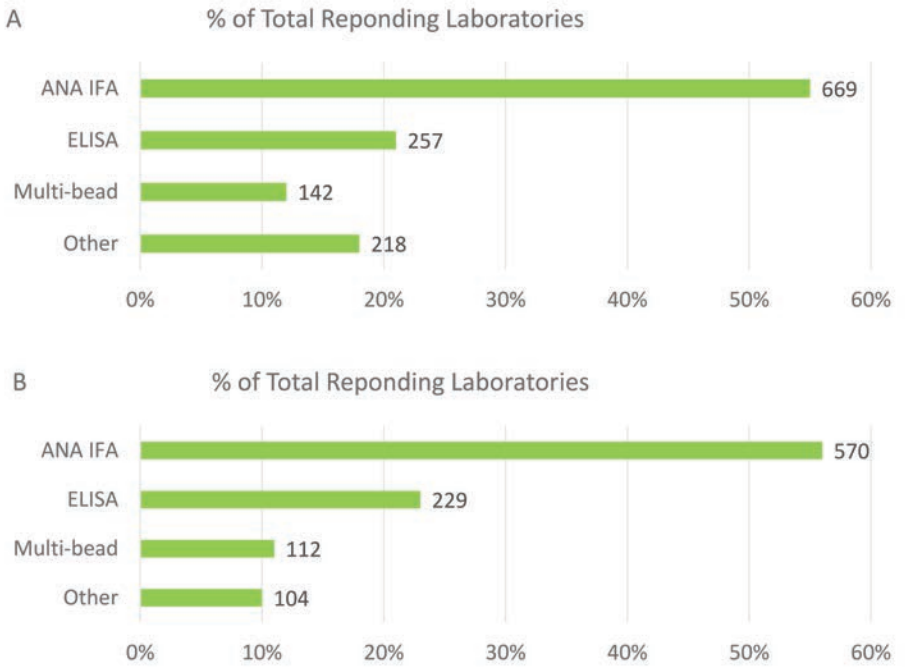

Figure 1. Distribution of (A) methods used among the 1206 responding laboratories (the total is > 100% because some laboratories used > 1 method), and (B) generic (preferred) methods among 1015 responding laboratories. Number of responding laboratories is shown to the right of each bar. ANA: antinuclear antibody; IFA: immunofluorescence assay.

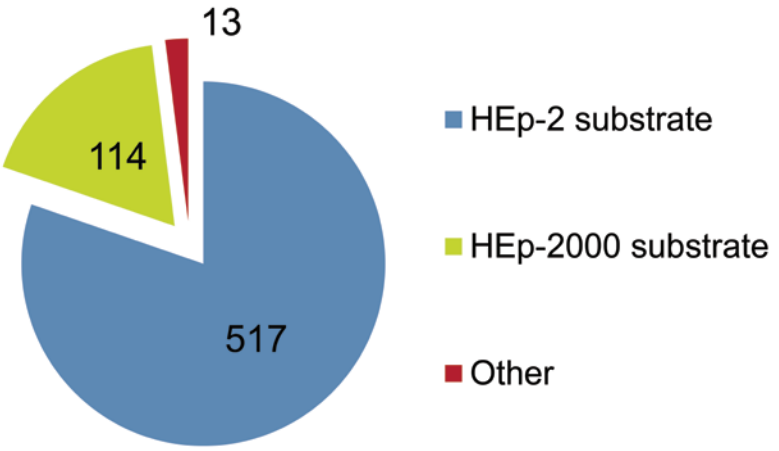

Figure 2. Distribution of substrate cell types used among the 644 responding laboratories.

Reporting. Screening serum titer varied among 637 respondents: < 1:40, 9 (1%); 1:40, 393 (62%); 1:80, 185 (29%); 1:100, 17 (3%); 1:160, 30 (5%), or other, 3 (< 1%). A positive test was reported at the traditional titer of 1:40 by only 315 (50%) of 628 respondents (Figure 3). There was a difference in practice between the US and international laboratories. In the US, screening was performed below 1:40 in 1%, at 1:40 in 72%, 1:80 in 23%, 1:100 in < 1%, 1:160 in 4%, and at another titer in < 1%. In contrast, in international laboratories, screening was performed below 1:40 in 2%, at 1:40 in 39%, 1:80 in 44%, 1:100 in 8%, 1:160 in 7%, and at another titer in < 1%. The highest titer reported by 563 respondents was < 1:640 (7%), 1:640 (24%), 1:800 (< 1%), 1:1000 (< 1%), 1:1024 (< 1%), 1:1240 (< 1%), 1:1250 (< 1%), 1:1280 (31%), 1:2040 (< 1%), 1:2320 (< 1%), 1:2560 (25%), 1:2580 (< 1%), 1:3120 (< 1%), 1:5000 (< 1%), 1:5120 (10%), and > 1:5120 (1%). Of 633 laboratories responding to a question about endpoint titration, 271 (43%) stated they routinely titered to endpoint, 143 (23%) titered to endpoint only upon request, and 219 (35%) never titered to endpoint.

Pattern reporting. Of the 644 laboratories performing ANA IFA, 634 (98%) reported an immunofluorescent pattern when present. Dual or multiple patterns were reported by 593 (92%) laboratories; however, 138 (23%) of 591 of these laboratories failed to report titers for each pattern.

DISCUSSION

Autoantibody targeting of the cell nucleus was discovered by Hargraves, *et al* in 1948 when they described the LE cell, a phagocytosed nucleus in a leukocyte[5]. Svec introduced spleen tissue substrate screening for ANA in 1967[6] and Yantorno, *et al* introduced mouse liver substrate for ANA testing in 1974[7]. In 1983, Kallenberg, *et al* described human fibroblast monolayers, HEp-2 cells, as superior to rat liver, based on the following: better recognition of distinct nuclear staining patterns, more convenient serum titrations, a cryostat not being necessary, the ability for multiple tests to be performed on 1 slide, and possible detection of significantly higher ANA titers in sera from patients with SLE and MCTD. In addition, they found that centromere antibodies were detected on HEp-2 cells, but not on rat liver sections[8].

Technological advancements[9,10,11,12,13], scarcity of skilled laboratory workforce capable of accurately and reproducibly reading HEp-2 cell IFA patterns and dilution titers[14], and the unmet need of having an ANA testing method that is easier to perform and shows a greater intra- and interlaboratory reproducibility than IFA, have all led to the development of ELISA using HEp-2 cell lysates as antigen for coating microtiter plate wells and other non-IFA methods[9,10,11,12,13,14]. However, ELISA using HEp-2 lysates as antigen could not fully control for

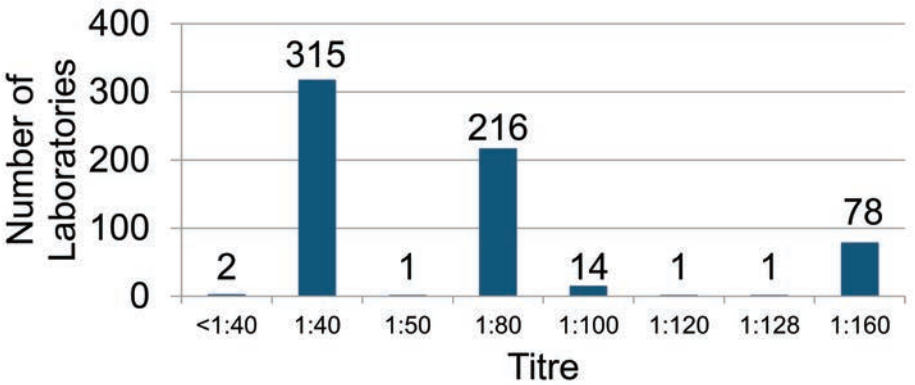

Figure 3. Positive titer cutoff for 628 responding laboratories.

concentration of components in cell lysates, especially minor components, across manufacturing lots[9,10]. Scarce cellular components may be underrepresented and autoantibodies to them remain undetected[10]. As purified or recombinant target antigens became available, they were used in combination as antigen in ELISA wells or on immunobeads. ELISA using combinations of purified or recombinant proteins as well as immunobead-based assays typically utilize a limited set of the more common antigenic targets. Further, the antigenic target is presented out of its cellular context. Epitopes displayed on a HEp-2 cell lysate ELISA microplate or on immunobeads may be different than what is presented in a cell substrate, where antigen more closely remains in its native configuration and intermolecular interactions in protein and nucleoprotein complexes are intact[15,16]. Bead-based technologies utilize an array of latex beads with magnetic cores; attached to the surface is a single antigen and chemical tag emitting light of a specific wavelength when lasered. By tagging different antigen beads with unique laser chemical emitters (Luminex technology), different beads may be tested together. Beads are incubated with patient serum, washed, then incubated with an antihuman IgG fluorescein–labeled antibody, washed, then each individual bead is enumerated for fluorescence intensity in a flow channel, much like a flow cytometer, and identified by its laser tag. This method allows for high throughput and minimal labor as the method is automated[17]. However, screening ANA by immunobead-based methods may underestimate positivity due to the limited number of antibody specificities detected compared to methods using cell substrates[1,9,16,18,19,20]. We found a difference between the United States and international laboratories. International laboratories were more likely to use ANA IFA on HEp-2 substrate whereas US laboratories had a higher penetration of ELISA or immunobead methods that can be readily automated.

The role of the ELISA and immunobead methods as a "screen" for ANA was questioned by an expert ad hoc committee of the ACR. Their recommendation that the ANA by IFA on HEp-2 cell substrate be the preferred method for ANA screening was accepted by the ACR Board of Governors in 2010[1,3]. Our data demonstrated that slightly more than half of testing laboratories used the IFA method with HEp-2 cells. ELISA using HEp-2 cell lysate as antigen does not control for concentration of antigens included and immunobead-based methods do not consist of many, including nucleolar, antigens as noted above[9,15,16]. A "negative" report may mislead clinicians into assuming these autoantibodies are absent. Many autoantibodies encountered in SSc, for example, are nucleolar such as PM/Scl, Th/To, and fibrillarin (U3-small nucleolar ribonucleoprotein), and recognition of their presence informs clinicians as to disease phenotype, prognosis, and management. For example, Th/To antibody positivity is associated with limited cutaneous SSc but also with interstitial lung disease and pulmonary hypertension that may not be clinically apparent at presentation. While localized skin manifestations may suggest to the clinician a good prognosis, presence of Th/To autoantibody flags the patient as high risk for significant cardiopulmonary morbidity[21,22]. The CLIA regulations governing the CAP laboratory proficiency audits requires a single proficiency survey regime for all ANA test methods. The limited specificities tested by immunobead methods has led to laboratory proficiency testing failures when blinded ANA test samples are positive for autoantibodies not tested in the immunobead method (e.g., nucleolar autoantibodies)[1,9,16,18]. Even within a given method, differences between manufacturers in preparation of antigen reagents may lead to proficiency test failures[13,23,24,25]. The ACR guidelines also recommended transparency about the method used[3]. Only approximately one-third of laboratories indicated the method used in the test name; 39% indicated the method used on reports. A fifth of all laboratories performing ANA IFA used non-HEp-2 substrate. Due to poor interlaboratory reproducibility of ANA IFA titers, the reliability of the reported ANA patterns and titers has come into question[13,23,24]. Proficiency survey samples distributed by the CAP are almost always selected as highly positive (with high titers), or negative with the IFA ANA method, to achieve conformity (> 80% agreement) and avoid nonconformity or mass failure of laboratories if the expected titers were close to the cutoff. In addition, high positivity rates of ANA IFA in low-risk populations have been reported and raise questions about clinical utility of positive ANA IFA results[25].

Within a given method, the procedure, cell substrate manufacture, and reagents used may affect results. The screening titer, cutoff titer required for positivity, and endpoint titer varied among laboratories. While ACR/European League Against Rheumatism (EULAR) classification criteria call for a positive ANA IFA at a titer of at least 1:80 for inclusion in SLE studies[26], many laboratories continue to screen ANA IFA at the traditional titer of 1:40. In our study, only half of the laboratories overall screened ANA IFA at the traditional titer of 1:40. However, in the US, a significant majority of laboratories still screen at the traditional titer of 1:40 (72%) compared to 1:80 (23%). In international laboratories, the screening titer is more evenly distributed between 1:40 (39%) and 1:80 (44%). The continued use of 1:40 screening titer in the US likely reflects historical practice, the analytical performance of the assay, limitations in detecting certain autoantibodies as noted below, and the diversity of diseases in which ANA may be encountered, rather than adherence to a disease specific classification schema (e.g., the ACR/EULAR SLE Classification Criteria[26]). However, there is a trend in both the US and international laboratories to use a higher titer for a positive test designation than the screening titer.

Therefore, it is critical for the clinician to know what titer cutoff was utilized; reporting a screen as "negative" without a reported screening titer may be misleading. For example, fixation of HEp-2 cells tends to deplete SSA Ro60, the autoantibody target in Sjögren syndrome[1,27]. Hence, low titer antibody to SSA below the threshold for "positivity" may be clinically significant[27]. A negative result for a screening at 1:160 may miss a positive SSA autoantibody evident on HEp-2 cells at 1:40 or 1:80. To address lowered SSA antigen density in fixed HEp2 cells, a

transfected HEp-2 cell line overexpressing the Ro60 antigen has been employed in some laboratories[27,28,29]; however, the intense staining of nuclei by SSA-positive sera may obscure staining patterns of other nuclear autoantibodies in positive sera with multiple autoantibody specificities. In contrast, titers of 1:40 or 1:80 for other autoantibodies may not be clinically significant. It is critical for the clinician to consider titer results within the clinical context[1,2].

It is also critical to understand what patterns are considered positive. Clinicians should understand whether their laboratory reports cytoplasmic-staining patterns as positive in an "antinuclear" antibody IFA. If not reported, cytoplasmic-staining autoantibodies such as anti-tRNA synthetase antibodies (seen in antisynthetase myositis syndromes), antimitochondrial antibody (seen in primary biliary cholangitis), or antiactin antibody (seen in autoimmune hepatitis) may be missed[13,30,31,32]. The International Consensus on ANA Patterns (ICAP), a subcommittee of the Autoantibody Standardization Committee of the International Union of Immunological Societies Quality Assessment and Standardization Committee affiliated with the Centers for Disease Control and Prevention, has defined ANA patterns and enumerated characteristics by which to identify each. Patterns were classified as nuclear, cytoplasmic, or mitotic. Patterns were further designated as either "competent" level, which should be recognized and reported by any laboratory performing routine clinical testing, or "expert" level, which may require higher technical skill to identify[33,34,35]. Competent nuclear patterns include common patterns such as homogeneous, speckled, dense fine speckled, centromere, discrete nuclear dots, and nucleolar. Further delineation of patterns, for example, nuclear speckled into fine speckled, large/coarse speckled, or topoisomerase I-like patterns, are designated as expert level. Similarly, nucleolar pattern delineation into homogeneous, clumpy, or punctate nucleolar are expert-level patterns. Subclassification of nuclear dots and cytoplasmic patterns are expert-level patterns. Nuclear envelop, nuclear pleomorphic, and all mitotic patterns are considered expert-level patterns[33,34,35,36]. Laboratories may not have the requisite skill set to report all patterns. Many of the expert patterns are rarely encountered and many of these lack commercially available monospecific assays to confirm suspected antigenic targets. Reeducation of a significant portion of the technical workforce will be required to expand laboratory capabilities in ANA IFA pattern recognition and is underway through ICAP, the College of American Pathology, and other laboratory-based professional societies.

The limitations of this study include the voluntary nature of participation and the participant response rate, but we believe the size of the sample makes respondents representative of laboratory practices. This is a study of variability in performance of ANA testing rather than an analysis of the analytical characteristics of the methodologies. Results were reported by laboratories over the signature of laboratory directors, but we have insufficient data on who performed the bench work, the level of expertise of the bench scientist/technologist, or the respondents' rationale for choosing 1 method or approach over the other.

ANA testing is a focus of the "choosing wisely" campaign to minimize inappropriate laboratory testing[37]. Improving clinician understanding of what is being ordered, how the test is performed, and what results mean will promote appropriate ordering. We recommend that the laboratory indicate the methodology employed in the test name and on the result report. For ANA IFA, the cell substrate and manufacturer, screening titer, positive titer cutoff, and patterns reported should be communicated. All patterns recognized in a sample should be reported including nuclear, cytoplasmic, and mitotic patterns. Dual patterns should be titered separately and reported. ICAP provides standardized definitions of patterns and multiple examples of each pattern on their Website (www.anapatterns.org), a useful training tool for clinicians as well as laboratory personnel[36]. While commercial automated platforms for image acquisition and pattern interpretation have been introduced, the number of patterns recognized is limited and technologists are still required to interpret the images, as reflected in our survey data[38]. For ANA screening by any non-IFA method such as ELISA or immunobead assays, the specificities detectable should be defined and communicated clearly in the test report. Failure to communicate to clinicians what the specific laboratory practices are may lead to clinical misinterpretation of results, delayed diagnosis, or inappropriate therapy.

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ONLINE SUPPLEMENT

Supplementary material accompanies the online version of this article.

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