Variability in Testing for Antineutrophil Cytoplasmic Antibodies

A Survey of Participants in the College of American Pathologists Proficiency Testing Program

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Context.—Variability in testing for antineutrophil cytoplasmic antibodies (ANCAs) contributes to confusion and controversy related to testing for vasculitis and other ANCA-associated diseases.

Objectives.—To survey laboratory testing practices regarding ANCA testing and to investigate differences in testing algorithms.

Design.—Supplemental questions were sent to the 333 laboratories participating in the College of American Pathologists proficiency testing program for ANCA as part of the Special Immunology S2 Survey.

Results.—A total of 315 laboratories submitted responses to the supplemental questions. Only 88 of 315 participants (28%) reported using a combination of indirect immunofluorescence (IFA) and enzyme immunoassay (EIA) techniques as recommended by current guidelines, with a few additional labs using IFA and multiplex bead assay as an acceptable alternative to EIA. Other labs reported using only IFA, EIA, or multiplex bead assays.

Conclusions.—A wide variety of testing algorithms are in use for ANCA testing despite evidence to suggest that a combination of IFA and EIA testing provides the most comprehensive information. Laboratories should inform clinicians clearly about testing practices and utility of testing in specific disease states.

Laboratory evaluation for the presence of antineutrophil cytoplasmic antibodies (ANCAs) is used diagnostically in the evaluation of patients suspected of having systemic vasculitis.1–4 The main utility in ANCA testing is for small-vessel vasculitides, including microscopic polyangiitis; eosinophilic granulomatosis with polyangiitis (previously known as Churg-Strauss syndrome); and small and medium vasculitides, such as granulomatosis with polyangiitis (previously known as Wegener granulomatosis). Together, these diseases are known as ANCA-associated vasculitides. Some drugs are associated with positive ANCA results with or without clinical vasculitis, such as propylthiouracil, methimazole, and carbimazole.5–7

Antineutrophil cytoplasmic antibodies are not specific for defined syndromes nosologically classed as vasculitis. Positive results are associated with autoimmune diseases, such as rheumatoid arthritis, scleroderma, systemic lupus erythematosus, and others.8–10 Positive results are seen in many patients with ulcerative colitis (but less commonly in Crohn disease) and primary sclerosing cholangitis.11,12 Additionally, cystic fibrosis patients commonly have a positive ANCA test result resulting from antibodies to bactericidal/permeability-increasing protein.13 Chronic bacterial infections and chronic liver disease have also been associated with positive ANCA test results.14,15

The classic method for detection of ANCAs uses an indirect immunofluorescence assay (IFA) technique where patient serum is incubated on a human neutrophil substrate followed by application of anti-human antibodies conjugated to fluorescein. The cells are reviewed with a fluorescence microscope. In positive cases, 2 main fluorescent patterns will be detected in ethanol-fixed neutrophils: one that reacts with the azurophilic granules distributed throughout the cytoplasm (cytoplasmic pattern, called cANCA) and one that reacts only with antigens located in the cytoplasm surrounding the nucleus (perinuclear pattern, called pANCA). The pANCA pattern results from ethanol-induced leaching of cationic proteins from the azurophilic granules that then collect around the negatively charged nucleus. In formalin-fixed neutrophils, cationic proteins are cross-linked and remain in the azurophilic granules; the immunofluorescent patterns of both cANCA and pANCA reactive sera seen on formalin-fixed cells, respectively, are cytoplasm-
mic granule staining. In most cases, cANCA results from proteinase 3 antibodies (PR3). Perinuclear pattern ANCA results from myeloperoxidase antibodies (MPOs) and occasionally leukocyte elastase or azurocidin.16,17 Enzyme-linked immunoassays (EIAs) for detection of PR3 and MPO antibodies are also available.

An international consensus statement on ANCA in 1999 recommended that screening be performed with IFA using both ethanol-fixed neutrophils and formalin-fixed neutrophils, and that EIA be performed on positive IFA samples.18 However, the consensus report recommended that EIA optimally be performed on all samples because the 2 methodologies are discordant in some cases. Current general practice is to screen with IFA and confirm positive results with EIA. A newer multiplex bead assay is also available which uses a combination of flow cytometry and antibody sandwich technique to detect antibodies to PR3 and MPO simultaneously in samples.19 Thus, the multiplex bead assay could be considered a variant of the EIA technique.

A third ANCA pattern, often noted as an atypical pattern, is rare in vasculitides and can show features of either cANCA (often with a diffuse or dull cytoplasmic pattern) or pANCA (often with intense nuclear membrane staining) on ethanol-fixed neutrophils. The specificities for these atypical ANCA patterns include lactoferrin, cathepsin G, elastase, lysozyme, bactericidal/permeability-increasing protein, catalase, alpha-enolase, actin, histone H1, high-mobility group protein-1, high-mobility group protein-2, and lamin B1, and the 50-kDa lamin-like neutrophil nuclear envelope protein.20 In addition, the presence of antinuclear antibodies (ANAs) may interfere with evaluation of IFA patterns. Use of both ethanol-fixed neutrophils and formalin-fixed neutrophils improves interpretation of ANCA patterns.21 In many cases, the atypical patterns are absent or decreased in formalin-fixed neutrophils. However, some difficulties arise in testing formalin-fixed neutrophils, including autofluorescence of cells.22 Indirect immunofluorescence testing for ANA using HEp-2 cell substrate can be used as a supplemental test to determine whether there is ANA interference.

Detection of ANCA is not always straightforward and use of these tests clinically is occasionally controversial. Issues regarding the methods of detection, laboratory testing algorithms, and clinical utility of serial testing are common. Additionally, the reported prevalence of ANCA in different disease states varies widely depending on disease stage, disease activity, prior therapy, and testing methods used.23 Laboratory testing practices vary because of the complexity of testing and possible interferences.

The Diagnostic Immunology Resource Committee acts as the expert scientific and educational resource for the College of American Pathologists (CAP) in diagnostic immunology and flow cytometry. Volunteer members from a variety of academic institutions and private laboratories oversee the proficiency testing (PT) for hundreds of analytes in our surveys. The primary purpose of this study was to examine the testing practices used by laboratories for detection of ANCA among the participants in the PT survey.

MATERIALS AND METHODS

A supplemental questionnaire was sent to laboratories participating in the ANCA PT survey (CAP Special Immunology S2 Survey). A total of 189 laboratories reported results for IFA on ethanol-fixed neutrophils, and 263 laboratories reported results for EIA testing (including laboratories that performed both). The brief results

RESULTS

A total of 333 unique laboratories reported ANCA results, and 315 laboratories responded to the questionnaire, a response rate of 95%. The first question asked laboratories about the algorithm used for ANCA testing. As seen in Figure 2, laboratories reported a variety of algorithms, including IFA only (84 of 315; 27%), EIA only (92 of 315; 29%), or a combination of both (88 of 315; 28%). A small percentage of laboratories (29 of 315; 9%) reported “Other” for their algorithm, which included laboratories running a combination of IFA and multiplex bead assay.

Several follow-up questions asked laboratories about the specificities of their testing algorithm. Of the total respondents, 190 laboratories reported using IFA in some capacity (shown in the Table), and of these, the vast majority (170 of 190; 89.5%) used both ethanol- and formalin-fixed neutrophil substrates. A total of 304 laboratories responded to the question about ANA being run on all samples (shown in the Table). A minority of these laboratories reported running ANA on all samples (43 of 304; 14%), and a similar minority (39 of 304; 13%) reported running ANA on pANCA cases only. The final question asked if laboratories reported results for atypical ANCA, to which 245 labs responded, and 135 laboratories (55%) responded affirmatively.

COMMENT

In this study, we sought to determine the current state of laboratory testing for ANCA. We found great variety in the testing algorithms used by laboratories in the detection of ANCA. More than a third reported using EIA or multiplex bead assays alone (114 of 315, or 36% of responses for either). Although these laboratories will detect the most common forms of ANCA associated with vasculitides, they will not be able to detect an atypical ANCA of any form. It is most likely that laboratories choose to perform this type of testing alone because EIA and multiplex bead assays can be run on automated platforms, and the EIA technique requires more specialized training of personnel to recognize the fluorescent patterns microscopically. One additional benefit of the EIA-only methods is that interference by the presence of an ANA will be very unlikely.

Laboratories reporting IFA testing only accounted for 84 of 315 total respondents (27%). Although the IFA remains the gold standard for ANCA testing, the EIA (and presumably the multiplex bead assay) are considered somewhat more sensitive for the presence of low-level anti-PR3 or anti-MPO antibodies. Laboratories performing IFA only will likely miss these low-level antibodies. It is unclear whether low-level antibody can be responsible for disease, but some studies have shown that testing with a combination of both IFA and EIA is more sensitive for ANCA-associated vasculitides than either alone. Clinicians ordering from these laboratories would either have to order the EIA separately, or wait for a negative result on the IFA and reflexively order the EIA if medically necessary.

Only 62 of 315 laboratories (20%) reported running IFA followed by EIA for positive cases, and an additional 16 of 315 laboratories (5%) reported running both IFA and EIA on
all cases. Either of these approaches fully complies with the International Consensus Statement on ANCA testing. Since this statement was published in 1999, current recommendations for testing continue to advocate for running both IFA and EIA. Interestingly, 10 of 315 laboratories (3%) reported running the EIA on all cases, and then running IFA on the negative cases (a reverse algorithm). Technically, this approach would also result in a similar sensitivity for disease. This reverse algorithm would provide labs with the ability to automate testing in a first-pass screening, and

Figure 1. Copy of supplemental questions and answer choices provided to participants in the S2 survey (Special Immunology). Abbreviations: ANCA, antineutrophil cytoplasmic antibodies; P-ANCA, perinuclear antineutrophil cytoplasmic antibodies.

Figure 2. Summary of testing algorithms used by laboratories responding to Supplemental Question 1. Abbreviations: ANCA, antineutrophil cytoplasmic antibodies; EIA, enzyme-linked immunoassay; IFA, indirect immunofluorescence assay.
Variability in Testing for ANCA

CONCLUSIONS

We believe optimal testing includes IFA on ethanol- and formalin-fixed neutrophil substrates and PR3 and MPO EIA

References


