Technical Article Series
Scleroderma ANA and Antibody Testing

ANA Testing Basics

The long-standing way of doing ANA testing is a method called indirect immunofluorescence (IFA). It is a time consuming, manual process subject to individual interpretation and is therefore fairly expensive. Depending on the lab, an ANA by IFA can detect up to 150 different types of antibodies (antigens). The main result of an ANA by IFA is called a titer (actually a dilution factor), but a staining pattern is also reported.

The titer reported with a positive ANA test is a measure of antibody levels, which is actually quite significant in some autoimmune diseases but not generally in Scleroderma. It should be reported as a dilution factor in a format such as 1:320, but sometimes you will only see the second number. The second number will always be a multiple of 40, so titer results might look like 1:40, 1:80, 1:160, 1:320, 1:640, etc. While technically a titer of 1:40 or above is normally considered positive, an ANA titer of 1:80 or less is not generally considered meaningful since a significant percentage of people in the general population will have a positive ANA at these levels with no clinical symptoms. When you get to a titer of 1:160 or higher, then this is usually an indication of an underlying autoimmune condition. Note that if you see an ANA test result that looks like a single low number (e.g., 3.8) then this indicates that the ANA test was done using an alternative testing method (ELISA or Multiplex). These alternative testing methods are discussed in some detail below.

The staining pattern can be somewhat subjective but overall it gives a rough idea of what type of antibody is detected. Some specific staining patterns are fairly specific to certain diseases (for example, a centromere pattern is highly correlated with limited Scleroderma). Other patterns are specific to Lupus or can occur with more than one autoimmune disease. In modern clinical practice, staining patterns are not considered to be definitive for specific diseases since detailed follow-on antibody testing is a much more accurate way of determining a likely diagnosis.

Scleroderma Antibody Testing

If the titer is high enough to suggest an autoimmune disease, then the next step is to run a separate diagnostic panel to try to identify the specific antibody that was detected by IFA. Note that some of these screening panels are specific for a particular autoimmune disease – Lupus, Scleroderma, or Mixed Connective Tissue Disease (MCTD). There is also often a general rheumatic disease screen panel.

Diagnostic testing panels use a variety of different testing methods, depending on the reference lab. However (and this is VERY important), a screening panel can *only* detect the specific antibodies that are included in the panel. While this sounds obvious, in fact the significance of this cannot be over stressed, as will be discussed below.

In recent years, the standard way of doing ANA testing has started to change. As was mentioned above, doing an ANA test by IFA is somewhat expensive since it requires human analysis and to some extent the results are subject to individual interpretation. Two alternative ways of doing ANA testing are rapidly becoming commonplace: ANA testing by a method called solid phase immunoassays (ELISA or EIA) or a related technique known as a Multiplex platform. These new methods are faster, cheaper, and eliminate the subjective interpretation problem. Unfortunately, they also introduce a significant major problem of false negative results – especially for patients with Scleroderm.

ANA testing by IFA tests for up to 150 different antibodies at one time. In contrast, typical ELISA testing tests for only 8 to 10 antibodies and typical Multiplex testing detects perhaps 11 to 13 different antibodies. These testing systems can be set up for different antibody panels. For example, an ELISA test for Lupus-related antibodies would test for a different set of 8 to 10 antibodies than an ELISA test for Scleroderma-related antibodies. Research shows that these two methods (ELISA and Multiplex) are very reliable in detecting the antibodies they are designed to test for.

Falsely Reported Negative ANA Result and Diagnostic Risk

If a physician suspects that her patient might have some form of Scleroderma based on the patient's symptoms, she would typically order an ANA test with reflex to a Scleroderma antibody panel. In the past, what the lab would have done is to first run an ANA test by IFA. If that result was positive, then a Scleroderma-specific antibody panel would be run to try to identify which (if any) Scleroderma-related antibodies the patient has.

Now, however, when the physician orders a Scleroderma ANA / antibody panel, the blood sample is usually tested using either ELISA or Multiplex instead of IFA. IF this test detects the presence of one of the 8 to 13 antibodies included in the panel, then in most cases a separate ANA by IFA will then be done to confirm the result, but also to measure the titer level and staining pattern (although the staining pattern is not particularly relevant at that point). However, if none of these 8 to 13 antibodies are detected, then the doctor receives a report that the ANA is negative.

Unfortunately, there is a big difference between testing for up to 150 antibodies and testing for up to 13 antibodies. A recent (2011) study looked at a typical Multiplex Scleroderma screening panel and determined that the antibodies included in the test missed *up to 43%* of Scleroderma patients that in fact tested positive by IFA because the panel simply did not test for antibodies that are now known to occur in a significant minority of Scleroderma patients. And, note that this was a Scleroderma-specific screening panel. If instead, the doctor was concerned that the patient might have *some* kind of autoimmune condition, but was not sure what type (potentially including Lupus, Scleroderma, and MCTD, for example), he would have not ordered a Scleroderma or Lupus or MCTD screening panel but would have instead ordered a general rheumatic screening panel. This time, however, even fewer disease specific antibodies would be tested for and the likelihood of a false negative ANA report would be even greater.

In order to get a better idea of how much of a problem this is in the real world, I recently reviewed about a half dozen national testing labs to see what types of ANA / autoimmune screening panels were offered by these labs and which specific antibodies were included in the Scleroderma screening panels in particular. Unfortunately, in this quick survey I found only one national reference lab (ARUP Labs) where their Scleroderma panel included all of the main antibodies that are now known to be associated with Scleroderma. Perhaps, not surprisingly, they also routinely do the initial ANA testing separately by IFA rather than Multiplex or ELISA. In all of the other labs that I reviewed, the Scleroderma screening panel excluded antibodies that represent more than 25% of the antibodies that are present in Scleroderma patients. The general rheumatic disease screening panels often do not always even include anti-centromere antibody testing, which was historically one of the two commonly recognized antibodies that have been identified for many years.

Discussion and Recommendations

Based on relatively recent research, any Scleroderma screening panel should definitely test for anti-RNA Polymerase III antibodies (this antibody is associated with one form of diffuse Scleroderma) in addition to the normal anti-Scl70 and anti-centromere antibodies, since this antibody is actually found in about 20% of Scleroderma patients. The next most common "new" Scleroderma-related antibody is anti-Th/To, which is associated with one form of limited Scleroderma and is found in up to 5% of Scleroderma patients. Any Scleroderma screening panel that includes at least these four antibodies should reliably detect the vast majority of Scleroderma patients. Unfortunately, it appears that at the present time, very few reference labs offer Scleroderma screening panels that include these newer antibody types. Hopefully, this will change in the near future.

The ultimate problem is that most physicians, especially primary care physicians, realistically have no idea how ANA testing or antibody screening panels are performed, so when they get a report of a negative ANA result after ordering a Scleroderma screening panel, most primary care doctors (and some rheumatologists without a lot of experience diagnosing Scleroderma patients) will believe the report and start looking for alternative explanations for a patient's symptoms. This can lead to significant delays in getting a correct diagnosis. Ironically, 15 years ago this would not have been as much of a problem since all ANA testing was initially done by IFA. (Although, to be fair, a Scleroderma screening panel 15 or 20 years ago would probably only have included testing for anti-Scl70 and anti-centromere antibodies since the newer Scleroderma-related antibodies had not yet been identified.)

Basically, the solution to this problem is very simple. If the physician orders an ANA test with reflex panel based on the patient's symptom profile and gets a negative result, then she needs to check to see if the ANA was run by IFA (the result would be reported as a titer). If not, then she needs to confirm this negative result by re-running the ANA test by IFA.

If this second ANA is also negative, then this significantly reduces the chance that the patient is dealing with an underlying autoimmune disorder. But it does not eliminate this possibility entirely since research studies report that between 2% and 10% of patients that are officially diagnosed with some form of Scleroderma based on their cluster of symptoms do have a negative ANA when done by IFA. In some cases, this negative result is false. For example, there are several different ways that ANA by IFA

testing is done, for example using human cells or rodent cells to perform the test. Generally, ANA testing by IFA is done using human cells (HEp-2 substrate), but if IFA testing is instead done with other types of cells and the patient has one of the common Scleroderma antibody types (anti-centromere or ACA), there is a significant chance that this antibody will not be detected and the overall ANA result will falsely be reported as negative. Some of these patients will eventually turn positive upon later retesting but some patients will permanently remain ANA negative. For this reason, it is important that the ordering provider either verify that the ANA by IFA is being done using HEp-2 substrate or if not sure, include in the screening a separate anti-centromere antibody test along with the ANA by IFA.

If, however, this second ANA test yields a positive result, then follow-up detailed testing for other Scleroderma-related antibodies not included in the original screening panel is the appropriate next step (this would also be the appropriate point to send the patient to a rheumatologist if one was not already part of the patient's care team).

The *American College of Rheumatology*, in a 2011 Position Statement, recommends that testing by IFA "should remain the gold standard for ANA testing". While that may be ideal, for cost reasons it is expected that initial ANA screening will increasingly be performed using new lower-cost methods such as ELISA or Multiplex. This in itself is not a problem since a significant percentage of Scleroderma patients will correctly test positive using these methods. However, because of the very real potential of a false negative ANA result that can potentially cause major delays in accurate diagnosis and early treatment, if the ordering physician suspects that an underlying autoimmune condition is the root of the patient's presenting symptoms, then it is very important that any negative result of an ANA test done by any method other than IFA be verified by re-testing for ANA using IFA.

References

American College of Rheumatology Position Statement. Methodology of testing for antinuclear antibodies. www.rheumatology.org/practice/ana_position_stmt.pdf. Approved by Board of Directors: Aug 2011.

Bonilla E, Francis L, Allam, F, Ogrinc M, Neupane H, Phillips PE, and Perl A. Immunofluorescence microscopy is superior to fluorescent beads for detection of antinuclear antibody reactivity in systemic lupus erythematosus patients. *Clin Immnol* 2007 124:18-21.

Rolling G. Challenges and Controversies in Anti-Nuclear Antibody Testing. *CLN News* Oct 2009: 34:10.

Rolling G. Antinuclear Antibody Testing Dilemmas, Does High Throughput Trump Sensitivity? *CLN News* Nov 2011: 37:11.

Shanmugam VK, Swistowski DR, Saddic N, Wang H, Steen VD. Comparison of indirect immunofluorescence and multiplex antinuclear antibody screening in systemic sclerosis. *Clinical Rheumatology* Oct 2011 30(10): 1363-1368

Ulvestad, E. Performance characteristics and clinical utility of a hybrid ELISA for detection of ANA. *Apmis* 2001 109:217-222.

Revision History

- **7/2014:** Initial version published