Contribution of Reflex MPO/PR3 Testing in Anti-neutrophil Cytoplasmic Auto-Antibodies Positive Sera: An Indian Perspective

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Abstract: Background: Anti-neutrophil cytoplasmic antibodies are serological markers of primary systemic vasculitis. Antigen-specific tests such as ELISA to detect antibodies directed against the myeloid enzymes, MPO and PR3 are essential in addition to screening by indirect immunofluorescence. Study was undertaken to 1) evaluate the diagnostic value of ANCA measurement by IIF, coupled with reflex anti-PR3 (for c-ANCA) and anti-MPO (for p-ANCA) ELISA for ANCA positive specimens, and 2) evaluate the utility of testing specimens simultaneously by all 3 tests, i.e. IIF, MPO and PR3 ELISA. Methods: The study was a retrospective study performed at a CAP and NABL accredited private reference laboratory in Mumbai in a community setting. A total of 23,920 serum specimens from patients with suspected small vessel vasculitis were tested for ANCA by IIF over a period of 5½ years. Of these, 855 specimens (3.57%) (Group I) had a pre request for anti-MPO or anti-PR3 ELISA following a positive IIF-p-ANCA pattern or IIF c-ANCA pattern respectively. Group II comprised another 52 specimens which had a pre-request for all 3 tests i.e. IIF, MPO and PR3 ELISA simultaneously, irrespective of the IIF positivity. Results: ANCA seroprevalence was 31.11% in group I with predominant p-ANCA pattern (65.41%), p-ANCA was more frequent in females and c-ANCA in males. Anti-MPO and anti-PR3 positivity was 23.56% and 36.95% respectively, highlighting the non-specific nature of IIF-ANCA. Anti-MPO & anti-PR3 positivity was lower in females (21.10% and 31.70%, respectively) as compared to males (27.69% and 41.17%, respectively). Simultaneous performance of the three tests in Group II did not yield any significant advantage over reflex testing. Conclusion: Utility of reflex testing of IIF-ANCA positive specimens to MPO/PR3 ELISA is reinforced in the Indian population. However, test requests for using the reflex algorithm are low. Prescribers must thus be encouraged to follow the reflex algorithm for patient benefit.

Key Words: Anti-neutrophil cytoplasmic antibodies, myeloid enzymes, MPO, PR3

Introduction:
Anti-neutrophil cytoplasmic antibodies (ANCA) directed against several myeloid enzymes are serological markers for patients with primary systemic vasculitis. Two major patterns can be distinguished by indirect immunofluorescence microscopy on neutrophils: cytoplasmic ANCA (c-ANCA) and perinuclear ANCA (p-ANCA). c-ANCA nearly always reacts with proteinase 3 (ANCA-PR3) and p-ANCA with myeloperoxidase (ANCA-MPO) by ELISA test. In atypical ANCA's the immunofluorescence test is positive (mostly p-ANCA), but the ELISA for both ANCA-MPO and ANCA-PR3 is negative. ANCA-PR3 positivity is predominantly found in patients with Wegener’s granulomatosis and microscopic polyangiitis. ANCA-MPO-positivity is found in patients with microscopic polyangiitis as well as in patients with other vasculitides and auto-immune conditions. Atypical ANCA's are seen in a wide variety of medical conditions. (1) Antigen-specific tests such as ELISA with purified antigen are required in addition to the screening by indirect immunofluorescence (IIF), as the finding of a diffuse c-ANCA or p-ANCA is not equivalent to the presence of antibodies directed against PR3 and MPO, respectively.
Especially p-ANCA lacks specificity as these antibodies can be found in many other conditions.(2) The purpose of the current study was to 1) evaluate the diagnostic value of ANCA measurement by the indirect immunofluorescence (IIF) test, coupled with reflex anti-PR3 (for c-ANCA) and anti-MPO (for p-ANCA) ELISA for every ANCA positive specimen, and 2) evaluate the utility of testing specimens simultaneously by all 3 tests, i.e. IIF, MPO ELISA and PR3 ELISA.

Methods

Study design: Retrospective data analysis at a CAP and NABL accredited private Reference Laboratory in Mumbai.


Patient type & specimen size: A total of 23,920 serum specimens from patients with suspected SVV were tested for ANCA during this period of 5½ years. Of these, 855 specimens (3.57%) had a pre-request to perform MPO or PR3 ELISA testing, in the event of a positive IIF-pANCA pattern or IIF-cANCA pattern respectively. These 855 specimens were designated as Group I. Group II consisted of a subset of 52 serum specimens which had a pre-request to perform IIF, MPO and PR3 ELISA simultaneously, irrespective of the IIF positivity & pattern. Tests used were as follows:

1. Indirect Immunofluorescence (IIF) Technique for detection of ANCA:
   ANCA IIF was performed using a commercial kit (NOVA Lite® ANCA, INOVA Diagnostics, San Diego, CA, USA). The ethanol-fixed human neutrophil substrate slides were incubated with patient serum starting at a dilution of 1:20, along with positive and negative controls per batch. The slides were then washed in phosphate-buffered saline (PBS) to remove excess serum and incubated with fluorescein-labelled anti-human IgG immunoglobulin antibodies. The unbound reagent was removed by washing. Slides were then examined by fluorescence microscopy, for ANCA staining patterns (c-ANCA and p-ANCA). Positive sera were titrated by testing further twofold serial dilutions until a negative reaction was reached. The titre was considered to be the reciprocal of the last dilution to give a positive reactivity.

2. Anti-MPO & anti-PR3 Enzyme Immunoassays:
   Quantitative anti-MPO and anti-PR3 tests were performed using commercial ELISA kit (QUANTA Lite®, INOVA Diagnostics, San Diego, CA, USA). The microtiter wells were pre-coated with human MPO or PR3 antigens. The calibrators, controls and diluted patient samples, were added to the wells and incubated. After washing the wells to remove all unbound proteins, the antigen-antibody complex was reacted with an enzyme-labelled secondary antibody. Excess unbound conjugate was removed by washing. The bound conjugate was visualized by addition of a substrate. The absorbance was measured photometrically using an ELISA plate reader. The reactivity of each sample was calculated by plotting a calibration curve and the level of anti-MPO or anti-PR3 antibody read directly from the calibration curve. The intensity of the colour obtained in each well is proportional to the concentration of autoantibody in the sample. The assay was calibrated in U/mL against an arbitrary reference calibrator, as no internationally recognised reference preparation was available. A positive MPO- or PR3-ANCA result was defined as >9 U/mL and >3.5 U/mL, respectively.

Results

Analysis of Group I: Requests for reflex Anti-MPO or Anti-PR3 ELISA testing in the event of a positive IIF-ANCA result, were very low (3.57%). A total of 855 serum samples were analyzed in this group. The ANCA seroprevalence was 31.11% with predominant p-ANCA pattern (65.41%) as shown in Chart 1. p-ANCA had a higher preponderance in females while c-ANCA had a higher preponderance in males. Only 23.56% of IIF-pANCA and 36.96% of IIF-cANCA positive specimens showed MPO and PR3 reactivity respectively.

Table 1: Simultaneous ANCA IIF and anti-MPO, anti-PR3 ELISA

<table>
<thead>
<tr>
<th>Total specimens for simultaneous testing (n=52)</th>
<th>MPO-ANCA</th>
<th>PR3-ANCA</th>
</tr>
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<tbody>
<tr>
<td>Positive</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Negative</td>
<td>3</td>
<td>3</td>
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</tbody>
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ANCA pattern (N=33)

Analysis of Group II: Simultaneous performance of the 3 tests i.e. IIF-ANCA, anti-MPO and anti-PR3 ELISA did not yield any significant advantage over reflex testing. Only 1 specimen was reactive for anti-PR3 antibodies, despite of being IIF-ANCA negative as shown in Table 1.

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</tr>
</tbody>
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ANCA pattern (N=33)

Discussion

ANCA are commonly found in many chronic inflammatory diseases such as rheumatoid arthritis, Felty’s syndrome, systemic lupus erythematosus, ulcerative colitis, chronic active hepatitis, primary sclerosing cholangitis, systemic HIV infection, active tuberculosis, cystic fibrosis, Sweet’s syndrome and subacute bacterial endocarditis.(3-5) In these conditions, ANCA are produced as an innate immune response to neutrophils that are constantly recruited to inflammatory sites, and are probably involved in the active removal of neutrophil debris.(6) Most of these conditions do not result in diagnostically important production of MPO- or PR3-ANCA. Hence, ELISA using purified MPO and PR3 antigens are useful in differentiating patients with recent onset SVV from those with other systemic inflammatory diseases.

The ‘International Consensus Statement on Testing and Reporting’ reiterates that ANCs associated with SVV should only be reported as positive if both the IIF test and ELISA for MPO- or PR3-ANCA are positive.(7) Data analysis in the current Indian study group reinforces this understanding.
The ANCA seroprevalence in the current study was consistent with that reported in literature.(8-10) It was also observed that MPO/PR3 antigen specificity was lower among females as compared to the males. This highlights the fact that ANCA IIF positivity in females could very often be non-specific due to the higher prevalence of other autoimmune conditions.

The low percentage (3.57%) of requests for reflex testing, points to the need of encouraging physicians to use the reflex algorithm for accurate diagnosis. To counter the issues with IIF-ANCA non-specificity, the ‘International Consensus Statement on Testing and Reporting of ANCA’ recommends addition of comments on the IIF reports, stating the need to perform anti-PR3 and anti-MPO ELISAs for confirming antigen specificities, when IIF results have been disclosed without testing the latter.(7)

Being a single diagnostic laboratory, it was not possible to seek detailed clinical history of differential diagnoses in the current study group. This remained the major limitation of the current study. Nevertheless, ANCA testing should be restricted in patients with a clinical picture consistent with ANCA-associated diseases. This reduces the total number of tests performed, minimizes false positive results and laboratory charges and prevents unnecessary and potentially harmful diagnostic and therapeutic interventions.(9)

Simultaneous performance of the 3 tests in Group II only had the advantage of a faster reporting turn-around-time. The single specimen reactive for Anti-PR3 ELISA could be a false positive result since assay used was a direct PR3 ELISA. False positive anti-PR3 ELISA results have also been reported in another study.(11)

Conclusion
The current study reinforces the utility of reflex testing of ANCA-IIF positive specimens to MPO/PR3 ELISA assays in the Indian population. Simultaneous performance of the IIF-ANCA, Anti-MPO & Anti-PR3 do not yield any advantage over reflex testing. Clearly, more physicians must be encouraged to follow the reflex algorithm for accurate diagnosis and effective patient management.

Conflicts of interest: None

References