Clinical Utility of Screening for Antinuclear Antibodies by Enzyme Immunoassay — A Preliminary Study

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Abstract

Aims of the study: To evaluate the advantages and reliability of screening for antinuclear antibodies (ANA) by enzyme immunoassay (ELISA).

Methodology: Sera from 96 patients comprising 51 with systemic lupus erythematosus (SLE), 11 with other systemic rheumatological diseases (SRD) and 34 with various other diseases (non-SRD) were tested using a commercial ELISA kit (ANA-Ease, Genesis Biotechnology, U.K.). These sera consisted of 53 immunofluorescence assay (IFA) ANA-positive and 43 IFA ANA-negative samples.

Results: We observed that when compared to the IFA for ANA the sensitivity, specificity, predictive values for positives (PPV) and negatives (NPV) of ELISA were 90.7%, 85.7%, 89.1% and 87.8% respectively. Exclusion of borderline ELISA positive by slightly raising the cut-off optical density (OD) increased the specificity and PPV to 93.1%, and 94.1% respectively. Importantly, none of the non-SRD sera were positive when this higher cut-off was used. ELISA was noted to be strongly positive in three IFA ANA-negative SLE patients. However there was no correlation between the ELISA ANA semi-quantitative index and the IFA ANA titers.

Conclusions: ELISA appears to be suitable as a preliminary screening test for ANA. An appropriate cut-off should be identified to segregate low positive samples that could be false-positives. Nevertheless, IF will need to be performed to estimate the titers, identify patterns of ANA positive samples and confirm results of low positive “gray-zone” samples and ELISA negative sera from patients with a high index of clinical suspicion of SLE.

INTRODUCTION

The indirect immunofluorescence assay (IFA) performed on substrate slides of cultured human epithelioid (HEp-2) cells is currently considered to be the gold standard for the detection of antinuclear antibodies (ANA). Recently, broad-specific, generic enzyme immunoassay (ELISA) kits for the detection of ANA have become commercially available. ELISA being more economical than IF has gained considerable popularity. However the reliability and clinical utility of these ELISA kits has not been adequately studied particularly in the Indian context and many clinicians are not familiar with their performance characteristics.

The present study was carried out to assess the usefulness of ELISA as a screening modality for detection of ANA. The purpose was not to replace IF, which has several advantages, but to evaluate the reliability of ELISA as a diagnostic tool and identify the factors that should be considered when interpreting results when ELISA has been used.

MATERIAL AND METHODS

Study group

A total of 96 consecutive patients with results of IF ANA were studied. Of these 53 were IF ANA positive at titers of 1:40 strong 2+ positivity or greater and 43 were IF ANA negative. Serum samples of these patients had been received at our laboratory for various immunological tests. However due to lack of facilities for IF ANA against Hep-2 cells at our centre the samples had been simultaneously sent to any one of two other laboratories for this test. All clinical records of the patients including hematological, biochemical, serological...
and radiological investigations were assessed. The study group comprised 51 patients with systemic lupus erythematosus (SLE), diagnosed according to the American College of Rheumatology classification criteria,4 11 patients with other systemic rheumatic diseases (other SRD) and 34 patients with various disorders excluding systemic rheumatic diseases (non-SRD) wherein the clinical features and ancillary investigations did not satisfy the ARA criteria for a diagnosis of SRD. The group of other SRD consisted of five patients with rheumatoid arthritis (RA), four with mixed connective tissue diseases (MCTD) and two with scleroderma, while the final diagnosis in the non-SRD group was primary glomerulonephritides in nine patients, cerebrovascular accidents and venous thrombosis in eight patients each, tuberculosis in three, primary antiphospholipid antibody syndrome in two and recurrent pregnancy losses, polymyositis, leucocytoclastic vasculitis and essential hypertension in one patient each.

**Methodology**

All the sera had been stored at -80°C till the time of testing. The technician performing the test was not informed about the IF ANA test results. The sera were tested for ANA in our laboratory using ANA-Ease (Genesis Biotechnology Ltd., U.K.), a commercial ELISA screening kit. The antigen coated onto the polystyrene microtiter wells of this kit is a mixture of antigens extracted from Hep-2 cell nuclei and comprises of double stranded DNA, histones, SS-A/Ro, SS-B/La, Sm, Sm/RNP, Sc1 70, centromere Jo 1. The test procedure as recommended by the manufacturer was followed. The positive and negative control and cut-off calibrator were included in each assay. The optical density (OD) was measured on an ELISA reader at 450 nm wavelength. A semi-quantitative index, (ANA index) based on the ratio of the differences between the OD of sample and negative control to the difference between OD of cut-off calibrator and negative control was calculated as suggested by the manufacturer. Results were considered to be positive when the ANA index was greater than 1.0 (A). We also used a higher cut-off ANA index viz. greater than 2.0 (B) to exclude low positive samples which might not be clinically significant.

**Data analysis**

The sensitivity, specificity, positive and negative predictive values (PPV) and (NPV) were calculated using formulae of Vecchio.5 These values were calculated for both the ANA indices used. Since IF is considered to be the gold standard for ANA testing we considered IF ANA positive samples as true positives and IF ANA negative samples as true negative. However, though SLE patients who are IF ANA negative are rare, a few such cases have been reported in literature.6,7 Hence we also evaluated the sensitivity of the ELISA for the detection of ANA in clinically diagnosed cases of SLE. This group included nine IF ANA negative patients.

**RESULTS**

A total of 55 samples were found to be positive by the ELISA kit when the ANA index of greater than 1.0 (A) was chosen as the cut-off for positivity. Out of the 53 IF ANA positive samples 49 (92.5%) were true positive by the ELISA and 37 (86.1%) of the 43 IF ANA negative samples were true negatives. The distribution of true and false positives and negatives in the various diseases is depicted in Table 1. When the ANA index of greater than 2.0 (B) was considered as the cut-off, the percentage of true negatives increased to 93.02% however the true positivity decreased to 88.7%. The ANA index ranged from 1.04 to 505.0 in the patients who were ELISA positive. The lowest index (1.04) was observed in a patient with a CVA who had a negative IF ANA. In the patients with positive IF-ANA the ELISA ANA index ranged from 3.59 to 505.0. However, in individual cases we observed no correlation between the ANA index obtained by the ELISA test and the titers of the ANA as observed by the IF.

The sensitivity, specificity, PPV and NPV for the ELISA as compared to the IF is depicted in Table 2. Raising the ELISA-ANA cut-off index to greater than 2.0 (B) slightly decreased the sensitivity of the ELISA as compared to IF however the specificity and PPV increased considerably.

In our study there were nine patients who fulfilled at least four of the ARA criteria4 for SLE but had a negative ANA by IF. A false positive ANA by ELISA was noted in four of these nine patients. The ANA index (1.12) was marginally positive in one patient but was clearly in the positive range in the other three (ANA indices = 4.78, 9.04 and 39.0 respectively). One of these three patients died and the autopsy revealed lupus nephritis class V and a lupus pneumonitis. A false positive ELISA ANA was also noted in one patient with scleroderma and one with a CVA. The positivity was only marginal (index = 1.26 and 1.04 respectively).

**Table 1 : Disease-wise distribution of ELISA-ANA true and false positive and negatives as compared to IF-ANA**

<table>
<thead>
<tr>
<th>Study group</th>
<th>ANA index</th>
<th>True positive</th>
<th>True negative</th>
<th>False positive</th>
<th>False negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLE (n=51)</td>
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<td></td>
</tr>
<tr>
<td>A</td>
<td>41</td>
<td>5</td>
<td>4</td>
<td>1</td>
<td></td>
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<tr>
<td>B</td>
<td>40</td>
<td>6</td>
<td>3</td>
<td>2</td>
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<tr>
<td>Rheumatoid arthritis (n=5)</td>
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<td></td>
<td></td>
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<tr>
<td>A</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>1</td>
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<tr>
<td>MCTD (n=4)</td>
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<tr>
<td>A</td>
<td>4</td>
<td>0</td>
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<td>0</td>
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<tr>
<td>B</td>
<td>4</td>
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<td>Scleroderma (n=2)</td>
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<tr>
<td>B</td>
<td>1</td>
<td>1</td>
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<td>0</td>
<td></td>
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<td>Primary glomerulonephritis</td>
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<td>(n=9)</td>
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<td>8</td>
<td>0</td>
<td>0</td>
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<tr>
<td>B</td>
<td>0</td>
<td>8</td>
<td>0</td>
<td>1</td>
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<td>Cerebrovascular accident</td>
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<td>(n=8)</td>
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<tr>
<td>A</td>
<td>0</td>
<td>7</td>
<td>1</td>
<td>0</td>
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<td>B</td>
<td>0</td>
<td>8</td>
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<td>Venous thrombosis</td>
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<td>B</td>
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<td>8</td>
<td>0</td>
<td>0</td>
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<td>Tuberculosis (n=3)</td>
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<td>A</td>
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<td>2</td>
<td>0</td>
<td>1</td>
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<tr>
<td>B</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td></td>
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<tr>
<td>Others* (n=6)</td>
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<td></td>
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<tr>
<td>A</td>
<td>1</td>
<td>4</td>
<td>0</td>
<td>1**</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>4</td>
<td>0</td>
<td>1**</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations : A = cut-off ANA index for judging positivity of ELISA-ANA greater than 1.0, B = ANA cut-off index greater than 2.0, Others* = Primary antiphospholipid syndrome (2), recurrent pregnancy losses (1), essential hypertension (1), leucocytoclastic vasculitis (1). ** = patient with recurrent pregnancy losses.
respectively) and the samples would be rated as negative at cut-off ‘B’. Specific tests for individual ANA have not been done in any of these patients hence we have not been able to evaluate the possible basis for the positive ELISA.

When we considered clinically diagnosed cases of SLE as positive controls we observed that the sensitivity of IF for the detection of ANA was 82.4% as against 88.2% and 84.3% (positivity cut-off ‘A’ and ‘B’ respectively) for the ELISA. The specificity, as observed in the negative control group of non-SRD, was 88.2% for IF while for ELISA it was 91.2% and 97.0% (positivity cut-off ‘A’ and ‘B’ respectively).

Lastly, the cost of an ELISA ANA test in our study was approximately one-third the cost of an IF ANA test.

**DISCUSSION**

Results of the ANA test are often useful in the diagnosis of auto-immune diseases, particularly SLE, however they can, occasionally, be misleading.1,2 Only a few studies8-11 have compared ELISA and IF for the detection of ANA. One of these observed variability in the sensitivity and specificity of ELISA.8 Another10 noted significant discrepancies between commercial IF and ELISA kits for the detection of ANA, while other studies9,11 found ELISA to be a useful modality. Thus whether the ELISA assay for ANA is a suitable alternative to the IF is still debated.

In the present study we observed that the ELISA kit for ANA showed a reasonably good sensitivity and PPV when compared to the IF (Table 2). Homburger et al11 had suggested that the use of different cut-off values could define significantly positive samples. We observed that raising the cut-off index marginally excluded some ‘false-positive’ samples and increased the specificity and PPV considerably (Table 2). Importantly none of the patients with non-SRD were positive when this higher cut-off was used.

It is well established that the results of autoantibody tests including ANA have only a supportive value and that the diagnosis of SLE and other systemic auto-immune diseases is based primarily on the presence of a set of defined clinical manifestations.4 We had therefore also considered patients with a clinical diagnosis of SLE as positive controls. We noted that the sensitivity of ELISA in our study group was slightly higher as compared to the sensitivity of IF ANA. Homburger et al11 had reported that in their study 100% of patients with clinically diagnosed SRD were ANA positive by ELISA while 95.4% were positive by IF. Technical factors, including variations in the substrate and subjective variability in interpretation, may underlie the lower sensitivity of IF as compared to ELISA.

A major disadvantage of the ELISA observed in our study was the lack of correlation of the ANA semi-quantitative index with IF ANA antibody titers. Probable causes for the discordance include the use of stored sera, differences in the substrate other technique related variables. Further studies using specific assays for individual ANA will need to be performed to understand this discrepancy. Studies comparing ELISA with IF, using larger numbers of patients and controls, as also studies evaluating various commercial ELISA kits are also necessary.

Thus to conclude, it appears that the ELISA assay for ANA is suitable as a preliminary investigation to exclude negative ANA sera. Being technically simpler, less subjective, and faster it could be particularly useful in laboratories screening large numbers of samples as many amongst these might be ANA negative. It is also more economical than the IF and this fact has important bearing in our country where IF may often be unaffordable or not feasible.

However the limitations of ELISA should be borne in mind. We suggest that low positive samples should be considered to be lying in the ‘gray zone’ and that each laboratory should identify a suitable cut-off ANA index which would segregate these samples from significantly positive ones. Depicted in Fig. 1 is an algorithm for assessing samples tested for ANA by ELISA. Retesting by the standard IF should be done for all low positive ‘gray zone’ samples for confirmation of ANA positivity and for all other positive samples to determine the strength of the antibody. Needless to say, the IF is also essential for identification of the distinctive patterns which have important clinical implications as these cannot be visualized or extrapolated by ELISA. Lastly, retesting by IF to confirm ANA negativity should be done in all ANA negative patients whenever there is a strong clinical suspicion of an SRD.

**REFERENCES**


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The following members were awarded the Fellowship of ICP at Hyderabad APICON 2004

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