INTRODUCTION

Connective tissue diseases are a group of disorders that involve multiple body systems and exhibit a wide spectrum of clinical manifestations. They share certain common features which include inflammation of skin, joints and other structures rich in connective tissue along with altered patterns of immunoregulation such as production of autoantibodies and abnormalities of cell mediated immunity. The term “auto-immune rheumatic disease” (ARD) is preferable to the older term “connective tissue disease” because the clinical effects of ARD are not limited to connective tissues only. The diagnosis of ARD is arrived at taking into consideration several clinical and laboratory criteria because these diseases are not organ-specific and have no single pathognomonic finding. Each ARD may be associated with different autoantibody types. Presence of these autoantibodies in a person is used as an aid to the diagnosis of autoimmune diseases. Anti-nuclear antibodies (ANA) refers to immunoglobulins that react against different autologous nuclear [eg. anti-double stranded deoxyribonucleic acid (dsDNA), anti-soluble...]

ABSTRACT

Background: Detection of antinuclear antibody (ANA) is used as one of the diagnostic criteria for autoimmune rheumatic diseases (ARD). Both indirect immunofluorescence (IIF) and enzyme linked immunosorbent assay (ELISA) methods are used for this purpose. However, there are lack of data comparing these two tests from India.

Methods: We prospectively studied 294 patients clinically suspected to be having ARD between April 2012 and September 2013. They were tested for ANA by IIF and ELISA methods. Representative samples positive by both the tests were processed again by a line immunoassay test to detect the specific antinuclear antibodies. Considering the IIF results as the ‘gold standard’, the utility of ELISA for ANA detection was analyzed.

Results: Of the 294 samples processed, 181 (61.5%) were from female patients. By IIF 30% of samples in males and 40.3% sample in females tested positive. We found ELISA to have a poor sensitivity (45.8%) but good specificity (99.5%). The positive predictive value for ELISA were 98% and negative predictive value 76.2% respectively. Forty four samples positive by both IIF and ELISA were tested by Western blot to detect individual autoantibodies. Of these, only 24 samples showed the presence of one or more bands, while the remaining 20 (45.4%) were negative by line immunoassay. In our study anti-nuclear ribonucleoprotein/Smith was the most common ANA detected.

Conclusions: The poor sensitivity raises concerns regarding the practice of initial screening for ANA by ELISA.

Key Words: Anti nuclear antibody, ELISA, Indirect immunofluorescence, Line immunoassay


INTRODUCTION

Connective tissue diseases are a group of disorders that involve multiple body systems and exhibit a wide spectrum of clinical manifestations. They share certain common features which include inflammation of skin, joints and other structures rich in connective tissue along with altered patterns of immunoregulation such as production of autoantibodies and abnormalities of cell mediated immunity. The term “auto-immune rheumatic disease” (ARD) is preferable to the older term “connective tissue disease” because the clinical effects of ARD are not limited to connective tissues only. The diagnosis of ARD is arrived at taking into consideration several clinical and laboratory criteria because these diseases are not organ-specific and have no single pathognomonic finding. Each ARD may be associated with different autoantibody types. Presence of these autoantibodies in a person is used as an aid to the diagnosis of autoimmune diseases. Anti-nuclear antibodies (ANA) refers to immunoglobulins that react against different autologous nuclear [eg. anti-double stranded deoxyribonucleic acid (dsDNA), anti-soluble...]

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substance A or Sjogren’s Syndrome A/Ro (SSA/Ro), and cytoplasmic components [aminoacyl transfer ribonucleic acid (tRNA) synthetase (Jo-1), mitochondria, etc]. Thus ANA usually target specific antigens in the nuclear part of the cells, and can sometimes show affinity against all types of subcellular structures and cell organelles, including the cytoplasm, nucleoli, or cell surfaces. ANA can be detected in several ARDs. Although a battery of laboratory tools are available for detecting ANA, indirect immunofluorescence antinuclear antibody (IIF) test, fluorescent antinuclear antibody (FANA) test and enzyme immunoassay (EIA) / enzyme linked immunosorbent assay (ELISA) are commonly used in day-to-day practice. IIF test is currently considered to be the gold standard for detecting ANA in clinical practice. By using human epithelial-2(HEp-2) cells as the substrate, IIF test permits the detection of antibodies to more than 30 different nuclear and cytoplasmic antigens comprising more than 50 autoantibodies.

Because of the ease of performing the test ANA detection by ELISA has often been used for screening purposes. Other main methods used for detection of specific antibodies include gel precipitation assays, passive haemagglutination, Western blot, multiple immunoassay, flow cytometry and antigen microassay.

This study was undertaken to assess usefulness of ELISA for detecting ANA compared to the gold standard of indirect immunofluorescent test and identification of specific important antinuclear autoantibodies by line immunoassay.

**MATERIALS AND METHODS**

We prospectively studied 294 patients clinically suspected to have ARD who presented to the Rheumatology Clinic at the Sri Venkateswara Institute of Medical Sciences (SVIMS), Tirupati, whose samples were submitted to the Department of Microbiology SVIMS Tirupati, for testing for ANA during the period April 2012 to September 2013. The study was approved by the Institutional Ethical Committee.

All the serum samples were kept at –20 °C until they were processed and were tested by indirect immunofluorescence antinuclear antibody test (Aeskuslides ANA HEP-2, Aesku Diagnostics, Germany) and ELISA was performed using Auto Stat™ Anti-Nuclear Antibody (ANA) screen (Hycor Biomedical Inc. USA). For ELISA, serum samples showing ANA concentration greater than 23 IU/mL were taken as positive as recommended by the kit manufacturer. Representative samples positive by both the tests were processed again by line immunoassay test to detect the specific antinuclear antibodies using Euroline ANA Profile 3 (Euroimmun, Germany). It can simultaneously detect antibodies to nuclear ribonucleoprotein / Smith (nRNP / Sm), Smith (Sm), soluble substance A or Sjogren’s syndrome A, Robert-52 (SSA, Ro-52), soluble substance B or Sjogren’s syndrome B (SSB), DNA topoisomerase 1 or scleroderma-70 (Scl-70), polymyositis-scleroderma (PM-Scl), proliferating cell nuclear antigen (PCNA), cytoplasmic histidyl tRNA synthetase (Jo-1), centromere protein-B (CENP-B), double stranded deoxyribonucleic acid (dsDNA), nucleosomes, histones, ribosomal protein-P, anti-mitochondrial antibodies (AMA-M2). The kit manufacturers’ instructions were followed for all the tests. The sensitivity, specificity, positive predictive value (PPV) and the negative predictive value (NPV) of ELISA were calculated considering IIF as the “gold standard”.

**RESULTS**

Of the 294 samples studied, 113 (38.4%) were obtained from male patients and 181 (61.5%) were from female patients. Among males, 34
(30%) tested positive by IIF; in females, 73 (40.3%) tested positive by IIF. Majority of the samples (n=274, 93.1%) were from patients in the age group of 10-59 years. Peak incidence of ANA positivity (29.9%) was observed in 30-39 years of age (Table 1).

Table 1: Age-wise distribution of ANA positive samples by IIF

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Samples studied No. (%)</th>
<th>Positive by IIF No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 - 9</td>
<td>1 (0.3)</td>
<td>1 (0.9)</td>
</tr>
<tr>
<td>10 - 19</td>
<td>33 (11.2)</td>
<td>11 (10.2)</td>
</tr>
<tr>
<td>20 - 29</td>
<td>48 (16.3)</td>
<td>20 (18.6)</td>
</tr>
<tr>
<td>30 - 39</td>
<td>80 (27.2)</td>
<td>32 (29.9)</td>
</tr>
<tr>
<td>40 - 49</td>
<td>77 (26.1)</td>
<td>22 (20.5)</td>
</tr>
<tr>
<td>50 - 59</td>
<td>36 (12.2)</td>
<td>14 (13.0)</td>
</tr>
<tr>
<td>60 - 69</td>
<td>12 (4.0)</td>
<td>4 (3.7)</td>
</tr>
<tr>
<td>70 - 79</td>
<td>7 (2.3)</td>
<td>3 (2.8)</td>
</tr>
<tr>
<td>Total</td>
<td>294</td>
<td>107</td>
</tr>
</tbody>
</table>

ANA = anti-nuclear antibodies; IIF = indirect immunofluorescence

Of the 50 blood samples positive by ELISA, all but one were positive by IIF. Two hundred and forty four samples were negative by ELISA, of which 58 (23.8%) tested positive by IIF, while 186 (76.2%) were found to be true negatives. Thus, IIF could pick up 23.8% additional positives which could not be detected by ELISA. On the other hand, IIF missed only one sample which was positive by ELISA, but could correctly detect the antibodies in all the other 49 samples. Thus ELISA has got poor sensitivity [45.8%; 95% confidence intervals (CI) 36.1 - 55.7] but good specificity 99.5%; 95% CI 97.0 - 99.9. The PPV and NPV for ELISA were 98% (95% CI 89.3 - 99.7) of 76.2% (95% CI 70.4 - 81.4) respectively.

Forty four representative samples positive by both IIF and ELISA were tested by line immunoassay to detect individual autoantibodies. Of these, only 24 samples showed the presence of one or more bands, while the rest 20 (45.4%) were negative by line immunoassay. In our study anti nRNP/Sm was the most common ANA detected (n=11) alone or in combination with other antibodies, followed by antibodies against SS-A (n=7).

**DISCUSSION**

Autoimmune diseases of all organ sites and systems affect approximately 8% of the population, 78% of whom are women. There is a consistent female preponderance for the ARD; ranging form 3:1 in systemic sclerosis to 9:1 in systemic lupus erythematosus (SLE). In our study, the ratio of female to male in clinically suspected cases was 1.6:1, and similarly ANA positivity was more in females (40.3%) compared to males (30%). The likely explanation for this female preponderance is probably related to exogenous endogenous hormonal changes. The X-chromosome contains a number of both sex-related and immune-related genes that determine immune tolerance and sex hormone levels.

There are also some indications that sex chromosomes may play a part in contributing to disease onset or severity.

In our study majority of the samples (93.1%) were from patients in age group of 10-59 years and we found the peak incidence of ANA positivity of 29.9% in 30-39 years of age. Age at onset varies widely among ARDs and so do their manifestations. For example, 65% patients with SLE start manifesting their symptoms aged 16-55 years while Sjögren’s...
syndrome is considered to be more prevalent in women between ages 45 and 50.\textsuperscript{11}

In our study we evaluated the performance of ELISA in the detection of ANA considering IIF as ‘gold standard’. The sensitivity and specificity of ELISA in our study were 45.8\% and 99.5\% respectively. A study from Bangladesh\textsuperscript{12} found low sensitivity of 55\% with ELISA, when compared with IIF for ANA detection in childhood SLE cases which is comparable with our study. However, in another study\textsuperscript{13} four ANA ELISA kits were evaluated and a high sensitivity ranging from 90\% to 97\% and specificities ranging from 36\% to 94\% were observed when compared to IIF in patient with SLE. One explanation for the high sensitivity observed in this study\textsuperscript{13} may be the use of clinically defined samples. Another study\textsuperscript{14} reported the performance of five ELISA kits and comparing them with IIF and found that the sensitivity and specificity of different ELISA kits ranged from 69.5\% to 97.7\% and 81.4\% to 97.9\% respectively. We found a similar high specificity for ELISA in our study. An explanation for low sensitivity in our study may be due to the use of imported kits which gave a high cut-off concentration levels to differentiate a positive and a negative sample. It appears logical that whenever imported kits are used it may be necessary to define a cut off concentration based on local population studies. The other more likely explanation may be due to the limited number of antigens used in the ELISA which may not reflect the autoantibody patterns of the local population. There may also be racial and/or ethnic differences in the autoantibody patterns found in different population groups.\textsuperscript{15}

We found that ELISA had a PPV and NPV of 98\% and 76.2\% respectively. A PPV and NPV of 100\% and 69\% respectively were reported for ELISA in one study.\textsuperscript{12} However, a PPV and NPV of ELISA as 35.8\% and 96.4\% respectively were reported in another study.\textsuperscript{13} This difference may be due to differences in study population and the inclusion criteria used in these studies.\textsuperscript{12,13}

The ELISA test formats use a cocktail of 10-14 antigens, depending on the commercial kit used. The antigens used may be native or recombinant.\textsuperscript{13} Almost all the kits contain disrupted human epithelial -2 (HEp-2) cells to supply the same antigenic sites found in HEp-2 IIF test. The use of this component remains controversial since some argue that it increases sensitivity while others maintain that it lowers specificity. On the other hand, IIF test formats use HEp-2 cells which contain high concentration of nuclear and cytoplasmic antigens which results in higher sensitivity. Thus it is understandable that ELISA with its limited content of antigens may fail to detect certain antibodies which can be detected by IIF.

We studied 44 representative samples by line immunoassay method to detect specific ANAs. Among these 44 samples, 24 (54.5\%) were detected positive for ANA showing either single type or a combination of two or more. In our study anti nRNP/Sm was the most common ANA detected (13 out of 44, 29.5\%) and ANA against SS-A antigen being the second ( 8 out of 44, 18.1\%). The Sm and nRNP antigens are targets for autoantibodies in SLE, and considered specific for this condition.\textsuperscript{17} However, Sm autoantibodies appear in only 20%-30\% of SLE patients while nRNP antibodies are found in 30%-40\% cases. Further, while anti-Sm is highly specific for SLE, anti-nRNP is relatively non-specific and may appear in various other conditions.\textsuperscript{18} Since both these autoantibodies are detected in the same band in the line immunoassay test, isolated presence of these bands in the absence of other SLE specific bands like anti-dsDNA may not be significant. The SS-A band, which was the second most common band pattern observed in our study is found in a variety of ARDs, particularly SLE and Sjögern
Co-relation with other band patterns is equally important in the interpretation of SS-A band like nRNP/Sm. Remaining 20 of the 44 samples did not show any banding by line immunoassay though they were positive by IIF. Probably these serum samples had some antibodies other than the most common ones coated on the strips used in line assay. The same issue was addressed in another study and the authors reported that 17.2% samples which were positive for ANA by IIF were negative by line immunoassay method. Hence, though by line assay we could differentiate ANA, it may sometimes miss the detection of rare ANAs. In our study line assay showed positivity for nRNP/Sm, SS-A, SS-B, dsDNA, AMA-M2, and nucleosomes in one sample, and nRNP/Sm, Sm, dsDNA, and nucleosomes in another sample. This shows that this method has the advantage to detect patients with overlap syndrome, giving more information about the ANA present in the patient sample than IIF, which needs greater expertise to detect different patterns of ANA in a single sample.

ELISA has gained popularity since by this method ANA assay can be performed on a multiplex platform, with larger number of samples in a quicker time and at a much lower cost. Moreover, IIF has a subjective interpretation which depends on the experience of the person performing the test while ELISA format has objective well defined parameters. For these reasons, ELISA is increasingly gaining popularity as an initial screening test, followed by confirmation of positive samples by IIF-ANA. However, low sensitivity and propensity for false-negative ELISA results raises concerns regarding the practice of using ELISA method for the detection of ANA as the initial screening method.

REFERENCES


