



## A COMPARATIVE STUDY OF ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) WITH IMMUNOFLUORESCENCE ASSAY (IFA) FOR THE DETECTION OF ANTI NUCLEAR ANTIBODIES

**PRIYADARSHINI SHANMUGAM, \*JEYA MEENAKSHISUNDARAM AND POTHINI J**

*Department of Microbiology, Chettinad hospital and research institute, Kanchipuram, Chennai, India*

### ABSTRACT

Anti-nuclear antibody (ANA) testing is widely used as a screening test in Autoimmune diseases. The most commonly used method for ANA testing is ANA-immunofluorescence assay (ANA-IFA). This study compared the diagnostic performance of Enzyme linked immuno sorbent assay (ELISA) with IFA for the detection of ANA. 125 samples were tested which included 75 samples from patients with clinical suspicion of autoimmune diseases and 25 positive and negative controls. Of the 75 test samples, IFA was positive in 31 (41.3%), negative in 44 (58.6 %) cases. ELISA was positive in 20 ( 26.6 %), negative in 55 (73.3%) cases. 13 (17.3%) samples were positive by both methods. 37 (49.3%) samples tested negative by both. 18 (24%) samples were IFA positive but ELISA negative. 7 samples (9.3%) were ELISA positive but IFA negative. Comparing with the gold standard IFA, the sensitivity of ELISA was 71.43% and the specificity was 86.84%. ANA by ELISA is less sensitive than IFA using the HEp 2 substrate. Positive and negative controls tested by both methods gave the expected results.

**KEYWORDS:** Autoimmune diseases, Antinuclear Antibody, Immunofluorescence, ELISA



**JEYA MEENAKSHISUNDARAM**

Department of Microbiology, Chettinad hospital and research institute,  
Kanchipuram. Chennai. India

## INTRODUCTION

Anti-nuclear antibodies (ANAs) are specific antibodies directed against a variety of nuclear antigens which have been detected in the serum of patients with many rheumatic and non rheumatic diseases<sup>1</sup>. While some ANAs occur in normal individuals, others are expressed almost exclusively in patients with rheumatic disease and serve as markers for diagnosis and prognosis<sup>2</sup>. These antibodies are involved not only in the disease pathogenesis, but they also constitute the basis for diagnosis and treatment<sup>1</sup>. The first description of ANA was made by Hargraves and colleagues in 1948 when they observed LE (lupus erythematosus) cells in the bone marrow of patients with SLE<sup>3</sup>. Anti-nuclear antibody (ANA) testing is widely used as a screening test in connective tissue diseases (CTD) such as systemic lupus erythematosus (SLE), scleroderma, CREST syndrome (calcinosis, Raynaud's phenomenon, esophageal motility abnormalities, sclerodactyly, and telangiectasia), Sjogren's syndrome, mixed connective tissue disease (MCTD), polymyositis, and dermatomyositis<sup>3</sup>. Their detection with high sensitivity and specificity is therefore of utmost importance. Various detection methods are in use and there is continuous pouring of newer techniques to facilitate diagnosis and therapeutic monitoring<sup>4</sup>. The most commonly used method for ANA testing is ANA-immunofluorescence assay (ANA-IFA)<sup>3</sup>. The IFA test is currently considered the "gold standard" for testing for ANAs in clinical practice. Immunofluorescence is a highly sensitive assay for the presence of antinuclear antibodies<sup>5</sup>. By using HEp-2 cells as the substrate, the IFA test permits the detection of antibodies to more than 30 different nuclear and cytoplasmic antigens comprising more than 50 autoantibodies<sup>6</sup>. Slides prepared from human epithelioid cells (HEp-2 cells) are used as a substrate is incubated with diluted serum. The presence of auto antibodies is detected by fluorescent anti immunoglobulin antibody, and characteristic morphologic patterns of fluorescent staining are observed<sup>3</sup>. As most ANAs have an IgG isotype, the detection of IgM and IgA will not improve sensitivity, but may reduce

the specificity<sup>6</sup>. Certain ANA-IFA patterns are associated with the presence of autoantibodies to certain nuclear antigens which in turn are associated with certain clinical states<sup>3</sup>. ANA IFA test results are usually reported based on 4 basic patterns: homogeneous, speckled, nucleolar, and centromere<sup>2</sup>. A diffuse or homogenous pattern is associated with such clinical states as SLE, rheumatoid arthritis, scleroderma, Sjogren's syndrome, and drug-induced lupus<sup>3</sup>. Disadvantages of immunofluorescence testing include the complexity of the procedure<sup>5</sup> and requirement of a fluorescent microscope, which may not be available in many laboratories. The ANA-IFA is a subjective assay requiring skilled personnel and is a manual assay with a significant amount of hands-on time<sup>3</sup>. Therefore, an ANA-enzyme immunoassay (ANA-EIA) is an attractive alternative to ANA-IFA<sup>3</sup>. The advantages of ELISA testing include the speed and simplicity of the assay<sup>5</sup> also mass produced coating antigen preparations may be more consistent from lot to lot than immunofluorescence cell substrates. Some ELISA assays approach immunofluorescence in their sensitivity and specificity for the identification of antinuclear antibodies<sup>6</sup>. No technical expertise is required to interpret the ELISA readings. Disadvantages of ELISA testing include reduced antigen diversity leading to decreased sensitivity<sup>5</sup>. The present study was done with the objective of detection of ANA by immunofluorescence assay using HEp-2 cell substrate and enzyme immunoassay in patients with suspected autoimmune diseases. This study also compared the diagnostic performance of the ELISA method for ANA screening compared with HEp-2 ANA using IFA testing.

## MATERIALS AND METHODS

This was a Cross sectional comparative study. The institutional ethical committee clearance was obtained prior to conducting the study. The blood samples were collected after obtaining informed consents from the patients. The number of

samples to be tested was decided after getting the statistician's opinion. A total of 125 samples were tested of which 75 samples were from patients with suspected autoimmune diseases. 25 samples collected from healthy individuals served as negative control and another 25 samples from patients with autoimmune conditions and previously shown to have detectable ANA served as positive control. Serum was separated by centrifugation and the serum samples were stored in the deep freezer at  $-20^{\circ}\text{C}$  till further testing. ANA Immunofluorescence Assay was performed using the Biosystems Immunofluorescence Kit. The procedure was carried out according to the kit manufacturer's instructions. The serum samples were diluted 1/80. 1 drop each of the Control and Test sera were placed on each slide well, ensuring to cover it completely. Serum anti nuclear antibodies bind to the corresponding antigens present in the HEp -2 cells coated on the slides. After an incubation of 30 minutes, the slide was drained and rinsed with phosphate buffered saline. The resulting antigen antibody complexes are detected by means of a fluorescein labeled anti human globulin. After a further incubation for 30 minutes, the slide was rinsed, mounting medium was added and then it was examined under the fluorescent microscope. The different patterns of fluorescence observed were Homogenous, Peripheral, Speckled, Nucleolar and centromere. ANA ELISA was performed according to the Kit (Calbiotech) Manufacturer's instructions. Briefly, diluted serum samples are added to wells coated with purified nuclear antigens. ANA specific antibody, if present, binds to the antigen. All the unbound material is washed away and the enzyme conjugate is added to the antigen antibody complex. After washing off the excess enzyme

conjugate, the plate is incubated to allow the hydrolysis of the substrate by the enzyme. The intensity of the color generated is proportional to the amount of the IgG specific antibody in the sample.

## RESULTS

125 samples were tested by both immunofluorescence (IFA) and ELISA. 75 samples belonged to the test group and 50 samples served as the control. In the test group, 18 were males and 57 were females. Of the 75 test samples from patients with suspected autoimmune diseases, IFA was positive in 31 (41.3%) cases and negative in 44 (58.6 %) cases. ELISA was positive in 20 ( 26.6 %), negative in 55 (73.3%) cases 13 samples (17.3%) were positive for ANA by both ELISA and IFA. 37 samples (49.3 %) tested negative by both ELISA and IFA. 18 ( 24%) samples were positive by IFA but negative by ELISA and 7 (9.3%) samples were positive by ELISA but negative by IFA. Table 1 shows the percentage positivity of ANA by ELISA and IFA. Table 2 shows the comparison between ANA detection by ELISA and IFA. Out of the 18 males, 5 (27%) were positive by IFA but out of the 57 females enrolled in the study, 19 (33%) were positive by IFA. The majority of the patients who tested positive for antinuclear antibodies belonged to the age group of 21 to 30 years followed by 41 to 50 years. All the 25 samples which were collected from healthy individuals were negative for ANA by ELISA and IFA. All the 25 samples which were known to be positive for anti nuclear antibodies tested positive by both IFA and ELISA.

**Table 1**  
**Results for ANA by IFA and ELISA**

Testing Method	Result	No of samples	Percentage
IFA	Positive	49	40%
	Negative	76	60%
ELISA	Positive	45	36%
	Negative	80	64%

**Table 2**  
**Comparative Analysis of ANA detection by ELISA and IFA**

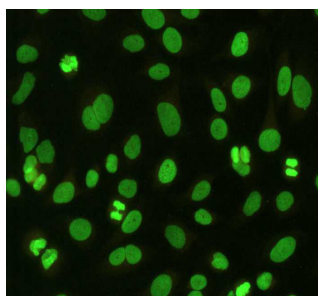
IFA	ELISA		TOTAL
	ANA (+)	ANA (-)	
ANA (+)	35 (a)	14 (b)	49
ANA (-)	10 (c)	66 (d)	76
TOTAL	45	80	125

The fluorescence patterns observed in the IFA positive samples were Homogenous in 26 samples, Speckled in 12 samples, Nucleolar in 5 samples, Cytoplasmic and Centromere in 2 samples each. The patterns showed varying intensity of 1<sup>+</sup>, 2<sup>+</sup>, 3<sup>+</sup> and 4<sup>+</sup>. Among the test

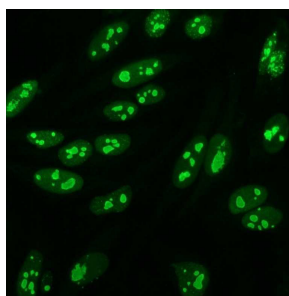
group, 5 were Homogenous, 5 were Speckled, 2 were Nucleolar, 2 were cytoplasmic. Among the control group, 11 were Homogenous, 12 were Speckled, 3 were Nucleolar, 2 were cytoplasmic and 2 were centromere patterns (Figures 1-6)

**Patterns of Immunofluorescence**

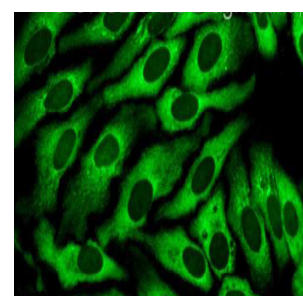
**Figure 1**  
**Homogenous**



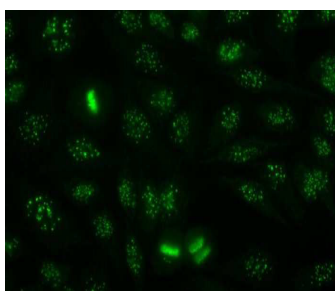
**Figure 2**  
**Nucleolar**



**Figure 3**  
**Cytoplasmic**



**Figure 4**  
**Centromere**



**Figure 5**  
**Speckled**

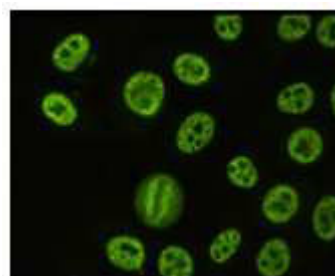
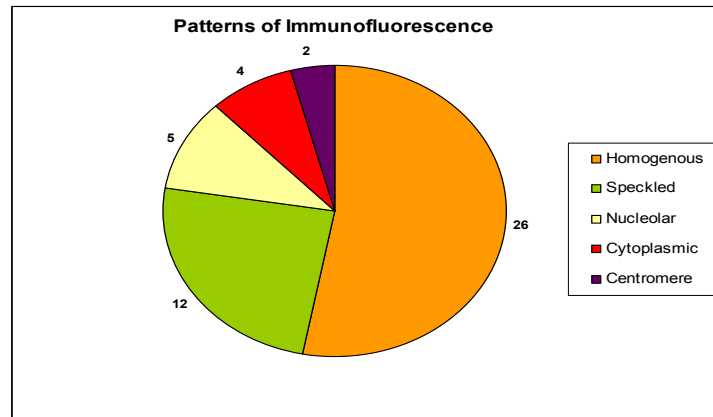


Figure 6



Sensitivity and specificity of ELISA in comparison to IFA was calculated using the following formulae : Sensitivity of ELISA =  $a / (a+b)$   $\{35/ 49 \times 100 = 71.43\%$  and Specificity of ELISA =  $d / (c+d)$   $\{66/ 76 \times 100 = 86.84\%$  Comparing ELISA with the gold standard IFA, the sensitivity of ELISA was found to be 71.43% and specificity of ELISA was 86.84%. The sensitivity of IFA was 98.3% and the specificity of IFA was 93%. The sensitivity and specificity of IFA was found to be greater than that of ELISA. IFA could detect Anti nuclear antibodies in samples that were negative by ELISA. Moreover samples that showed low titre value by ELISA were positive by IFA.

## DISCUSSION

In this study ANA positives were more among the females (33%) than the males (27%). This correlates with the findings of Hayashi <sup>7</sup> et al who reported that of the 111 patients with SLE, 104 were women and 7 were men, and the median age was 35 years. In the present study, most of the ANA positives belonged to the age group of 21 to 30 years followed by 41 to 50 years. ELISA for ANA was positive in 20 (26.6%) out of 75 patients suspected to have auto immune diseases and IFA was positive in 31 (41%) out of 75 patients. IFA showed more positives than the ELISA in the test group. This may be because ELISA was not able to detect all the types of antinuclear antibodies in the patient samples. Alternatively, it could be because

ELISA could not detect low positives. Gneiwek et al <sup>3</sup> reported that out of the 86 serum samples from Connective tissue disorder patients, 74 were positive by both ANA-IFA and ANA-EIA and 7 were negative by both methods. Four samples were positive for ANA-EIA but negative for ANA-IFA and one sample was negative for ANA-EIA but positive for ANA-IFA Therefore, the agreement between the two methods for samples from CTD patients was 94%. The findings of our study did not correlate with Gneiwek's study. A study in Bangladesh by Dipti et al <sup>1</sup> showed that ANA by IFA was positive in 27 out of 40 cases and ELISA was positive in 11 out of 40 patients. The findings of our study correlate with this study. A study by Fawcett et al <sup>8</sup> showed that IFA was positive in 157 out of a total of 184 samples tested whereas ELISA was positive in 95 samples only. This study also showed that ELISA testing did not correlate with IFA results. However, Gneiwek et al <sup>3</sup> inferred from their study that ANA-ELISA demonstrated equivalent sensitivity and somewhat higher specificity compared to ANA-IFA. In contrast to our study, Susan Copple et al <sup>6</sup> showed that IFA test had only 80% sensitivity for 30 confirmed SLE serum samples, while the Bio-Rad, Phadia, Aesku, and Inova ANA ELISAs demonstrated excellent screening sensitivities of 96.6%, 96.6%, 90%, and 96.6%, respectively. The lack of agreement between test methods may reflect differences in the array of antigens present in the various assays Different Fluorescent patterns observed were homogenous in 53.8%, speckled

in 23.07%, nucleolar in 11.53%, Cytoplasmic in 7.7% and Centromere in 3% of the IFA positive samples. Sebastian et al<sup>9</sup>, have observed homogenous pattern in 45.5%, speckled pattern

in 35.6% of the ANA positive samples. Sunitha et al<sup>10</sup> reported that a cytoplasmic granular pattern in 37 % of the samples and a homogenous pattern in 23 % of the samples.

## CONCLUSION

The lack of agreement between test methods may reflect differences in the array of antigens present in the various assays. Therefore, it can be concluded from this study that ANA by ELISA is less sensitive than IFA using the HEp 2 substrate. So it is recommended that ANA detection by IFA to be followed by the labs in screening for autoimmune diseases even though ANA detection by immunofluorescence is more expensive.

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