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Comparison of Different Methods for Detection of Auto Antibodies to Nuclear Antigens (ANA)

Dr. Subha M[1*], Dr. M Kavitha[2]
[1]Associate Professor of Microbiology, Madras Medical College, Chennai, Tamil Nadu, India
[2]Associate Professor of Microbiology, Kilpauk Medical College, Chennai, Tamil Nadu, India

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***Corresponding author**
Dr. Subha M

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Abstract: Antinuclear antibody testing is primarily applied as a diagnostic and prognostic tool for connective tissue disorders. Antinuclear antibodies can be detected by indirect immunofluorescence assay and enzyme immunoassay. This study was undertaken to compare the performances and cost effectiveness of immunofluorescence assay (IFA) using different substrates and enzyme immunoassay (EIA) in patient sera sent for routine testing of antinuclear antibodies.89 consecutive patient sera, clinically suspected to have **c**onnective tissue disorders, sent for routine antinuclear antibodies testing were analyzed by immunofluorescence assay using different substrates such as in-house mouse liver, BIOCHIP combination slide having mosaic HEp 20-10/ primate liver (Euroimmun) and EIA using Bindazyme™ ANA kit MK 200. Among the 89 sera, the number of sera positive for antinuclear antibodies were 33(37%) by IFA-mouse liver, 28(32%) by IFA- HEp20-10, 34 (38%) by IFA-primate liver and 19(21%) by EIA. When IFA-HEp 20-10 was taken as the reference method, the sensitivities, specificities, positive & negative predictive value of the other methods were as follows: 71%, 79%, 61%, 86% for IFA-mouse liver and 46%,90%,68%,79% for EIA. Immunofluorescence assay with in-house mouse liver substrate gives comparable results with commercial slides and is cost effective. Commercially available combination slides have performed well on all aspects; however they are not cost effective. EIA kit alone cannot be used as a screening test for antinuclear antibodies.

Keywords: Antinuclear antibodies, antinuclear antibody testing, Immunofluorescence assay, Enzyme immunoassay.

INTRODUCTION

Connective tissue diseases (CTD) are a group of autoimmune disorders, which are characterized by the presence of antinuclear antibodies (ANA) in the blood of patients [1]. Antinuclear antibodies (ANA) are a group of auto antibodies directed against the components of the cell nucleus such as nucleoproteins and nucleic acids. Presently the ANA have been categorized into two main groups: autoantibodies to DNA and histones which includes antibodies against single & double stranded DNA (ds DNA) and autoantibodies to extractable nuclear antigens (ENA) which include- Smith antigen(Sm), Ribonucleoproteins (RNP), SSA/Ro, SSB/La,Scl-70,Jo-1& PM1.

ANA can be used as a diagnostic and prognostic marker for the connective tissue diseases such as Systemic Lupus Erythematosus, Mixed Connective Tissue Disease, Systemic Sclerosis, Jorgen's syndrome [2]. ANA can be detected using several techniques like indirect immunofluorescence (IFA), enzyme immunoassay (EIA), immunoblot, immunodiffusion, line immunoassay, immune-precipitation and counter immunoelectrophoresis. The IFA test, a highly sensitive assay, is currently considered the "gold standard" for testing for ANAs in clinical practice [3]. Many laboratories have switched to solid phase immunoassays for screening of ANA as it can process large volume of clinical specimens, objective, less labor-intensive, and has the potential for automation [4]. In this study we compared the performances of immunofluorescence assay using different substrates and enzyme immunoassay for ANA testing in terms of sensitivity, specificity, ease of performance, cost factor and the time required for each technique.

MATERIALS AND METHODS

This was a cross sectional comparative study. A total of 89 consecutive samples were tested from patients with suspected autoimmune diseases. Serum was separated by centrifugation and the serum samples were stored in the deep freezer at – 20° C till further testing. All the sera samples were simultaneously tested by IFA with different substrates such as Biochip slide having mosaic HEp 20-10/ Primate liver

(EUROIMMUN) ,in-house mouse liver and EIA using Bindazyme™ ANA Screen Enzyme Immunoassay kit MK 200.

Procedure for ANA- IFA using Biochip slide

Procedure for ANA- IFA using Biochip slide having mosaic HEp 20-10/ primate liver substrates (EUROIMMUN GmbH Lubeck-Seekamp 31) was performed according to the manufacturer's instructions.

Procedure for ANA- IFA using in-house mouse liver substrate

ANA- IFA using in-house mouse liver substrate was done with samples and controls diluted to 1:10 and 1:40. 100µl of diluted sample was added over the smear and incubated for 30 minutes. The slides were washed twice using phosphate buffered saline (PBS) and shook well in the orbital shaker for 10 minutes. After air drying of the smears, 50µl of FITC conjugate (1:100 dilutions with PBS) was added over the smear and incubated for 30 minutes. The washing step was repeated as described previously. The slides were studied under NIKON fluorescent microscope after adding the mounting fluid over the smear.

ANA-EIA was done with Bindazyme™ ANA Screen Enzyme Immunoassay kit MK 200 (The Binding Site Ltd, Birmingham, England). The EIA plates are pre-coated with dsDNA, histones, SSA/Ro (60&52kD), SSB/La, Sm, Sm /RNP, Scl-70, Jo-1& centromere B.

RESULTS

Among the 89 sera, the number of sera positive for antinuclear antibodies was 37% by IFA-mouse liver at a reference range of 1:40, 32% by IFA-HEp20-10 at a reference range of 1:100, 38% by IFA-primate liver at a reference range of 1:100, and 21% by EIA. The manufacturer of ANA-EIA defines the result of <=10.0 as negative and >10.0 as positive.

Table 1: Number of positive and negative samples in each test

Method	No.of samples	No.of positive	No.of negative
IFA- mouse liver	89	33	56
IFA- HEp20-10/ primate liver	89	28	61
EIA	89	19	70

Of the 89 sera tested more number of females (71%) had ANA compared to males, of which 86% were in the age group of 16-45 years (Table 2).

The ANA titre assay using IFA HEp 20-10 (EUROIMMUN) was regarded as the reference method and the performances of other methods were evaluated. Sensitivity, specificity, positive predictive value and negative predictive value were calculated using standard formulae (Table 3).

Table-2: Age and sex wise distribution of positives in each test

Age	IFA mouse liver		IFA HEp20-10 / primate liver		EIA	
	M	F	M	F	M	F
0-15	1	2	1	2	1	2
16-30	4	7	2	9	2	8
31-45	3	11	1	8	2	3
46-60	2	-	1	2	-	1
>60	3	-	1	-	-	-
Total positives	13	20	6	21	5	14

Table-3: Performances of IFA using different substrates and EIA

Performance predictors	IFA with In-house mouse liver substrate	EIA
Sensitivity	71	46
Specificity	79	90
Positive predictive value	61	68
Negative predictive value	86	79

Sensitivity and specificity of IFA with in-house mouse liver substrate and EIA in comparison to IFA with HEp 20-10 substrate was calculated.

The methods were also evaluated on the basis of time and cost required for performing each method.

Table-4: Time and cost required by each method

Method	Cost per sample in Rs	Time required (min)
IFA in-house mouse liver	15	160
IFA- Comm HEp 20-10& primate liver	160	100
EIA	99	120

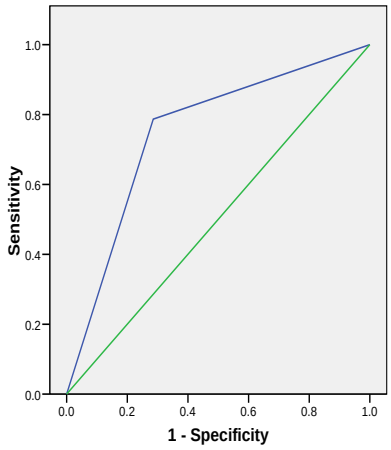
STATISTICAL ANALYSIS

Data were analyzed by statistical methods for the paired results from the ANA-IFA using mouse liver substrate and IFA-EIA against the IFA-HEp 20-10 substrate (reference method).The accuracy of tests were analyzed using receiver operating characteristic curve (ROC) methodology using SPSS for window(V:17) software.

IN HOUSE MICE - IFA - 1:40 * COMMERCIAL IFA - HEp 20-10 Crosstabulation

			COMMERCIAL IFA - HEp 20-10		
			POSITIVE	NEGATIVE	Total
IN HOUSE MICE - IFA - 1:40	POSITIVE	Count	20	13	33
		% of Total	22.5%	14.6%	37.1%
	NEGATIVE	Count	8	48	56
		% of Total	9.0%	53.9%	62.9%
Total		Count	28	61	89
		% of Total	31.5%	68.5%	100.0%

ROC Curve



Diagonal segments are produced by ties.

Area under the Curve

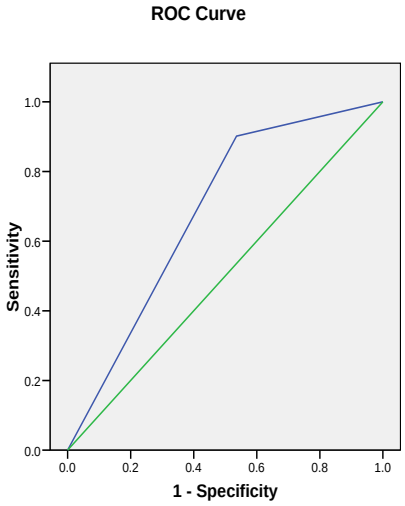
Test Result Variable(s): IN HOUSE MICE - IFA - 1:40

Area
.751

The test result variable(s): IN HOUSE MICE - IFA - 1:40 has At least one tie between the positive actual state group and the negative actual state group. Statistics may be biased.

ELISA * COMMERCIAL IFA - HEp 20-10 Crosstabulation

			COMMERCIAL IFA - HEp 20-10		
			POSITIVE	NEGATIVE	Total
ELISA	POSITIVE	Count	13	6	19
		% of Total	14.6%	6.7%	21.3%
	NEGATIVE	Count	15	55	70
		% of Total	16.9%	61.8%	78.7%
Total		Count	28	61	89
		% of Total	31.5%	68.5%	100.0%



Diagonal segments are produced by ties.

Area under the Curve

Test Result Variable(s): ELISA

Area
.683

The test result variable(s): ELISA has at least one tie Between the positive actual state group and the Negative actual state group. Statistics may be biased.

DISCUSSION

In this study ANA positives were more among the females (35.59%) than the males (20%). This correlates with the findings of Hayashi *et al*. [5]. In the present study, most of the ANA positives belonged to the age group of 16-30 years followed by 31-45 years similar to the study by Jeya *et al.* [6]. In this study IFA-HEp20-10 was taken as the reference method [7]. The sensitivity and specificity of IFA in-house mouse liver substrate was 71% and 79%. A study in Taiwan by Yang *et al*. [8] shows a sensitivity and specificity of 91.7% and 71.4% with IFA mouse liver cell substrate against IFA HEp 2 cell (CSI, USA). These discrepancies in ANA-IFA testing with different cellular substrates may be related to antigen antibody ratios, which may be less than optimal when cells of diverse tissues are used. Due to variable sensitivity with the substrate it is essential to report the type of substrate being used by the lab. However, the area under the ROC for in-house mouse liver is 0.75 which is comparable to commercial HEp 20-10 substrate.

In our study, EIA was found less sensitive and highly specific when compared to IFA similar to the findings of Hira-Kazal *et al*. [9]. In this study ANA by IFA was positive in 28 out of 89 cases and ELISA was positive in 19 out of 89 patients similar to the findings of the study in Bangladesh by Dipti *et al.* [10] that showed ANA was positive by IFA in 27 out of 40 cases and ELISA in 11 out of 40 patients. The reason may be that sera patients with systemic rheumatic diseases commonly have multiple autoantibodies including antibodies to antigen not found in the ANA-EIA. The number and type of antigens coated in an EIA plate vary with batch to batch and with manufacturers also. Thirdly, the equipments used for EIA vary in performance, with respect to intralaboratory and interlaboratory configuration. As a result, the results of the kits may be altered by equipment that are not similar to the equipment used by the manufacturers for production.

An important finding was that HEp 20-10 substrate slides were easier to interpret and gave a consistent pattern than the in-house mouse liver cell substrate. The IFA procedure is also comparatively shorter (100 min vs. 160min) using commercial slides than in-house slides. Even though IFA with commercial slides had so many advantages the cost per slide was very high (Rs.160) compared to IFA with in-house slides (Rs.15)

CONCLUSION

Immunofluorescence assay with in-house mouse liver gives comparable results with commercial slides and is cost effective. Commercially available combination slides for IFA have performed well in all aspects; however they are not cost effective. In this

study Enzyme Immunoassay kit gives least sensitivity when compared to other methods. While the findings of our study cannot be generalized it emphasizes that it alone cannot be used as a screening test for antinuclear antibodies. Therefore careful evaluation of the EIA kits is advisable before including these methods in the clinical and diagnostic testing.

ANA test results are an adjunct to the clinical diagnostic repertoire. Both IFA and EIA have their individual advantages and limitations. Hence an algorithm needs to be developed by the laboratory and the clinician to provide a logical sequence of screening and subsequent testing of ANA.

LIMITATION

The sample size was small due to financial constraint.

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