Original Research Article

ANA immunofluorescence versus profile-how well they perform in autoimmune diseases: an analysis of their clinical utility in a tertiary care centre

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ABSTRACT

Background: While Immunofluorescence assay remains the gold standard for the detection of ANA, Immunoprofile by ELISA is being increasingly utilized in view of easy availability and quick results. The study was done to find out whether ANA profile results are comparable with IFA.
Methods: About 100 patients who had undergone both immunofluorescence and Immunoprofile were included. Immunofluorescence correlation with profile and their correlation with the disease were analyzed; sensitivity, specificity and predictive values were calculated.
Results: ANA was positive in 78% by immunofluorescence; 73% by ANA profile. 22 patients in whom ANA IFA was negative were picked up by ANA profile. 27 patients who were not detected by ANA profile were tested positive by IFA. ANA testing by immuno profile had a sensitivity of 65% with a positive predictive value of 69% when compared with IFA. Immunofluorescence pattern and ANA profile correlated with the diagnosed disease in 63% and 49% respectively. Immunofluorescence pattern correlated with the ANA profile in only 35% of the study subjects. On correlation with the disease, ANA profile scored less compared to ANA-IFA with a sensitivity and specificity of 46% each; positive predictive value of 59%; negative predictive value of 33%. On analysis of individual disease, ANA profile is as good as IFA in SLE and scleroderma in terms of sensitivity. In Sjogren's syndrome and MCTD, specificity and positive predictive value of ANA profile is high.
Conclusions: ANA IFA performs better than immunoprofile in the diagnosis of autoimmune diseases.

Keywords: Antinuclear antibody, ANA immunoprofile, Immunofluorescence, Immunofluorescence versus profile

INTRODUCTION

Connective tissue diseases (CTD) are a group of autoimmune disorders characterized by the presence of antinuclear antibodies (ANA). The detection of these auto antibodies in the sera of the affected persons is very much essential to diagnose these diseases. The history of ANA testing dates back to 1948 with the observation of LE cell by Hargraves et al.[1] in the bone marrow of Systemic Lupus Erythematosus (SLE) patients. Although they are called as anti-nuclear antibodies, these antibodies exhibit reactivity not only against nuclei but also against various other cellular structures like cell surface, nucleoli and cytoplasm.[2] Despite its varied sensitivity in various

connective tissue diseases and false positivity results in numerous conditions, ANA is still widely used as a screening test for connective tissue disorders.

The two broad categories of ANA include a) auto antibodies against double stranded DNA (ds -DNA) seen in SLE and against histones seen in drug induced lupus. 3, 4 b) auto antibodies to extractable nuclear antigens (ENA) like antibody to Smith (Sm) antigen which is specific for SLE; other subtypes of ENAs include antibodies against ribonucleoproteins (RNP), SSA/Ro, SSB/La, Scl-70, Jo-1 and PM1.5 The sensitivity and specificity of ANA and its clinically important specific antigen subtypes is listed in Table 1.[6,7]

Table 1: The sensitivity and specificity of ANA and its clinically important antigenic subtypes.

	SLE	**93**	**57**
	Sjogren's syndrome	48	52
	Systemic sclerosis	85	54
ANA	Polymyositis/ dermatomyositis	61	63
	MCTD		
Antigen subtypes			
Anti -dsDNA	SLE	57	97
Anti - Sm	SLE	25-30	High
Anti -SS A/Ro	Sjogren's syndrome, Subacute cutaneous SLE, Neonatal lupus syndrome	8-70	87
Anti-SSB/La	Sjogren's syndrome, Subacute cutaneous SLE, Neonatal lupus syndrome	16-40	94
Anti-U3-RNP	Systemic sclerosis	12	96
Anti-centromere	Limited cutaneous systemic sclerosis	65	99.9
Scl-70	Systemic sclerosis	20	100
Jo-1	Polymyositis	30	95

The most commonly used laboratory tests to detect anti-nuclear antibodies include immunofluorescence (IF) assay and enzyme linked immunosorbent assay (ELISA). In immunofluorescence assay (IFA), the presence of a specific staining pattern indicates the presence of certain auto antibodies which in turn is associated with certain clinical states.[8-10] In antigen specific ELISA assays, we can detect the presence of various auto antibodies directed against specific antigens (ANA profile). Also, it is simple to perform, automated and does not require skilled personnel to interpret the results unlike IF assays.

The interpretation of ANA by immunofluorescence assay which is the gold standard is limited by the cost, requirement of skills and proper handling of the specimen. The detection of ANA by IFA does not always yield the specific clinical diagnosis; most often specific antibody assay is required to diagnose the clinical

syndrome. Also, the borderline ANA-IF staining (like 1+ positive; weak positive results) creates confusion which demands further diagnostic testing. It is noteworthy to understand that while certain diseases like MCTD can be diagnosed by the presence of a specific antibody anti - U1RNP, other autoimmune diseases have overlapping ANA IFA patterns and antibody profile. Detection of ANA by both immunofluorescence and ANA profile increases the cost and the time interval for diagnosis as each laboratory has its own turnaround times. Simply doing an ANA profile directly might cut short the time but sometimes it may not detect the antibodies when that specific antigen is not included in the kit in which case the diagnosis and thereby the treatment of the disease might get delayed. Each test has its own advantages and disadvantages. With this background, we intended to correlate the ANA results of 100 patients with autoimmune connective diseases who had simultaneously underwent ANA testing by both immunofluorescence (IF) and Immunoprofile (ANA profile) methods. These results were finally correlated with the disease diagnosed.

The aim of the study is to analyze the correlation between a) ANA- IFA pattern and ANA profile sub typing; b) ANA- IFA pattern and the disease diagnosed and c) ANA profile sub typing, and the disease diagnosed.

METHODS

The present study was a laboratory based observational study conducted in the adult patients who attended the Rheumatology OPD in a tertiary care centre in South India from August 2014 to June 2015. A total of 100 patients were included in the study.

Inclusion criteria

- Adults greater than 17 years and less than 60 years of age
- Who had underwent both ANA IFA and ANA profile tests.

Exclusion criteria

- Who had viral infections in the recent past
- Those on medications known to cause ANA positivity
- Those previously diagnosed to have rheumatic diseases
- Those above 60 years of age.

Thorough history and general physical examination were done for all the patients and diagnosis was made based on American College of Rheumatology (ACR) criteria.

Five milliliters venous blood was collected in a clean Wassermann tube, serum was separated from the clotted blood samples by centrifugation as per standard protocol. The serum was stored at -20°C until the time of assay. Determination of ANA was done by indirect

immunofluorescent techniques using HEp-2 Cell Line Substrate as per the standard recommendation; a titer of ≥1:100 was taken as positive for IF. ANA profile was done by using IMTEC-ANA-LIA MAXX for detecting antibodies against ds DNA, nucleosomes, histones, Sm D1, PCNA, SSA/Ro 60, SS A/Ro 52, SSB/La, CENP - B, Scl 70, U1sn RNP, AMA, M2, Jo 1, PM-Scl, Mi 2 and Ku as per the kit manual.

Table 2: Clinical significance of common Immunofluorescence patterns.

ANA –IF pattern	Antigen	Associated disease
Speckled	ENA, RNP, Sm, SSA/Ro, SSB/La, Scl-70, Jo-1, ribosomal-P	SLE, Mixed CTD, SS, Primary Sjogren's syndrome, PM
Homogenous	dsDNA, Histones	SLE, Drug induced SLE
Peripheral (rim)	RNP, Sm, SSA/Ro	SLE, SS
Nucleolar	Anti-PM-Scl, anti-RNA polymerase I-III, anti-U3-RNP, To RNP	SS, PM (polymyositis scleroderma)
Centromere	CENP A-E	Limited SS

The detection of ANA by profile method was compared with the standard IF assay. Also, the results of these two diagnostic assays (IFA and ANA profile) were subjected to correlation with the disease diagnosed. For example, when those patients diagnosed with SLE have homogenous pattern detected by IF method; double stranded DNA (dsDNA); histone antibodies in the ANA immunoprofile method, it is considered to be co-relating. The clinical significance of common ANA -IFA patterns and antibodies in ANA profile is listed in Table 2.

Statistical analysis

Descriptive statistics were reported using mean and standard deviation for the continuous variables, number and percentages for the categorical variables. Cross tabulation was done between ANA IFA (Gold standard) and ANA profile (ELISA). The sensitivity, specificity, positive and negative predictive values (PPV) and (NPV) were calculated. These values were calculated for both the ANA indices analysis.

RESULTS

Authors analyzed the ANA test results detected by both immunofluorescence and ANA profile method of 100 patients in whom diagnosis of rheumatic disease was made based on clinical and immunological criteria. Sjogren's syndrome and Mixed Connective tissue

Disease (MCTD) accounted for two-thirds of the study population; the distribution of various diseases in the study population is listed in Table 3. There were 72 females and 28 males with the mean age of 34.23±5.8 years. The female preponderance was noted in the disease subsets as well.

Table 3: Distribution of various rheumatic diseases in the study population.

Disease	Total	Female	Male
Systemic Lupus Erythematosus (SLE)	20	15	5
Sjogren's syndrome (SS)	32	21	11
Scleroderma	10	7	3
Mixed Connective tissue Disease (MCTD)	30	22	8
Myositis	4	3	1
CTD associated ILD (CTD – ILD)	4	4	0

ANA was positive in 78% of our subjects by immunofluorescence method and in 73% by ANA profile. The IF pattern observed were homogenous in 11; speckled in 48; nucleolar in 17; centromere and cytoplasmic pattern one in each. 51 patients (51%) were found to be positive by both IFA and immuno-profile methods. 22 patients in whom ANA IFA was negative were picked up by ANA profile. Also 27 patients who were not detected by ANA profile were tested positive by IFA method. We found ANA testing by immunoprofile has a sensitivity of 65% with a positive predictive value of 69% when compared to standard IFA method. The results were tabulated in Table 4. Specificity and negative predictive value could not be calculated since all our study subjects had established rheumatic autoimmune disease.

Table 4: Comparison of ANA IFA and ANA profile results.

	ANA IFA	
	Positive	Negative
ANA profile	51	22
	27	0
	78	22

Correlation of ANA results with the disease

In 63% of the subjects, the pattern observed in the immunofluorescence correlated with the diagnosed disease; whereas ANA profile correlated with the disease in 49%.

Both the tests correlated with the disease in 29 patients. On correlating IFA pattern with ANA profile, we found that IFA pattern correlated with the observed antibodies in ANA profile in only 35% of the study subjects.

On cross tabulation of correlation with the disease, we found that ANA profile scored less compared to ANA-IFA with a sensitivity and specificity of 46% each; positive predictive value of 59% and negative predictive value of 33% (Table 5).

Table 5: ANA profile vs. IFA correlation with the disease.

	Immunofluorescence pattern correlation with the disease	
ANA profile correlation with the disease	Present	Absent
	29	20
	34	17
	63	37

Sub analysis based on individual disease

On comparison of ANA results with respect to individual disease, we found that in SLE, ANA profile is as good as Immunofluorescence assay in picking up the disease with a sensitivity of 90% although positive predictive value (PPV) was 47%. Out of 10 patients in whom ANA-IFA was negative, 9 were picked up by ANA profile. We noted a high PPV of 82% in Sjogren's syndrome with a sensitivity of 64% when compared with IFA method. All the scleroderma patients were picked up by ANA profile while IFA method fails to detect the disease in one. The performance of ANA profile in other diseases is less compared to standard IF method. The results are tabulated in Table 6.

Table 6: Comparison of ANA immunofluorescence assay (IFA) and ANA profile results in disease subsets.

		ANA IFA					
		Positive	Negative	Sensitivity	Specificity	PPV	NPV
SLE	Positive	9	10	-	-	-	0
	Negative	1	0	90%	0	47%	0
Sjoren's syndrome	Positive	18	4	-	-	-	-
	Negative	10	0	64%	0	82%	0
Scleroderma	Positive	9	1	-	-	-	-
Myositis	Positive	1	2	-	-	-	-
	Negative	1	0	50%	0	33%	0
MCTD	Positive	13	4	-	-	-	-
	Negative	13	0	50%	0	76%	0
CTD	Positive	1	1	-	0	50%	0
	Negative	2	0	33%	-	-	-

(left axis label: ANA profile)

PPV - positive predictive value; NPV- Negative Predictive value

Table 7: ANA immunofluorescence assay (IFA) and ANA profile correlation with the disease.

			ANA IFA correlation with disease					
			Present	Absent	sensitivity	Specificity	PPV	NPV
	SLE	Present	8	10	80%	0	44%	0
		Absent	2	0	-	-	-	-
	Sjoren's syndrome	Present	8	2	44%	86%	80%	55%
		Absent	10	12	-	-	-	-
	Scleroderma	Present	5	3	100%	40%	62%	100%
		Absent	0	2	-	-	-	-
	Myositis	Present	1	2	50%	0	33%	0
		Absent	1	0	-	-	-	-
	MCTD	Present	6	2	24%	60%	75%	13%
		Absent	19	3	-	-	-	-
	CTD	Present	1	1	33%	0	50%	0
		Absent	2	0	-	-	-	-

(left axis label: ANA profile correlation with the disease)

PPV - positive predictive value; NPV- Negative Predictive value

Correlation with the disease

In SLE, ANA profile correlated well with the disease with a sensitivity of 80% and PPV of 44%. In Sjogren's syndrome, authors found that ANA profile scored well in ruling in the disease with a high specificity of 86% and NPV of 55%; also, its sensitivity is 44% with a very good PPV 80%. With respect to Scleroderma, ANA profile

performed well with 100% sensitivity and NPV; although specificity is 40%. Similarly, although sensitivity is low in MCTD, in 75% of the cases ANA profile is correct in predicting the disease. The results are tabulated in Table 7.

DISCUSSION

Very few investigations in the field of Medicine have such an important role in the diagnosis of diseases like the detection of anti-nuclear antibodies (ANA) for autoimmune diseases. Majority of referrals to a tertiary care centre for positive results following unnecessary ANA testing are often based on ELISA results rather than IFA; studies have revealed that majority of them are false positives.[11] Each method has its own advantages and disadvantages.

The present study analyzed the correlation between ANA results detected by IFA and ANA profile in 100 patients with autoimmune diseases.

There are many studies which had compared ANA results detected by IFA vs. ELISA in patients with suspected autoimmune disease.[12-14] But there were only few studies which had assessed the results in patients with established autoimmune disease.[15-17] The present study is one of its kind which had utilized both the tests (IFA and ANA profile) for establishing the diagnosis and then compared the performance of ANA profile with IFA pattern; correlated both the test results with the disease diagnosed.

Female preponderance is often observed in autoimmune diseases; the present study observed a female preponderance of 72% similar to that noted in other studies.[18-20]

ANA IFA was found positive in 78 patients (78%); of these ANA - IFA positives, 51 were found to be positive by ANA profile as well. The present study findings were similar to that observed by Latha M et al, who had reported that ANA IFA was positive in 71.3% out of 279 samples; 159(56.9%) were both ANA IF and Line Immuno assay (LIA) positive.[18] In another study conducted in East India, among 394 samples tested for both ANA IFA and ANA profile, 138 were ANA IFA positive (35.02%) in 1 in 40 dilution; of which 114 (82.6%) were also line immune assay positive.[19]

Positive ANA IFA with negative ANA profile is noted in 27% cases. This may be attributed to the fact that some rare auto antibodies may be missed by ANA profile if the kit fails to incorporate that specific antigenic substrate, which could be detected by IF staining. Review of the ANA pattern found in these 27 cases had shown speckled in 13; nucleolar in 12, homogenous and cytoplasmic pattern in 1 each. Similar observation was noted by Baronaite et al, who had studied the ANA results in 400 patients using IFA and Elisa immuno assay (EIA) techniques and found that the EIA method was less

reliable for assessing nuclear and speckled reactivity patterns.[13]

22 patients who were ANA IFA negative were picked up by ANA profile in the present study. Almost half of them had dsDNA antibodies (12 patients); Ro 52 and Scl 70 antibodies in 3; Sm antibodies in 3; nRNP/Sm+RibP in 3; PCNA/ histone antibodies in 1. The negative immunofluorescence could be because SSA and dsDNA antibodies were not detected by ANA -IFA method. The negative immunofluorescence may be due to the low concentration of auto antigens or its destruction during the preparation process.[21]

Surprisingly SLE, which is largely diagnosed by ANA positivity, would have been missed in 50 percent of the cases if only IFA assay is done. This showcases few interesting thoughts, where in the past, ANA negative lupus had been diagnosed based on simple assays, when this advanced ANA profile was not available. This was also noted in other studies, where significant number of IFA assays missed reactivity to dsDNA which was picked by the sub typing analysis.[13,18]

Similar to present study observation, there are various studies, which have shown inconsistencies between ANA IFA and ANA profile results. Latha M et al, noted that 40 ANA IFA positive samples (20.1%) were negative by ANA profile. Of the 80 ANA-IFA negative samples, 14 (17.5%) were detected to be positive by IF method.[18] In one study, positive result by IFA was noted in 17.3 % cases although they were negative for line immunoassay; The pattern observed in that study was mostly homogenous (66%) while in present study it was speckled followed by nucleolar pattern. In the same study, 14.8% of the ANA negative samples were tested positive by line immunoassay.[19] Sebastian et al, noted that 17.5% of ANA IFA positive samples were not detected by LIA method while 13.5% which were negative by IF method were found positive by LIA.[22]

This observation stresses the importance of further investigation with immunoprofile although IFA is negative if the clinical features are strongly suggestive in a given clinical setting. So, it is noteworthy to understand that there are certain auto antibodies which may escape staining by IFA method while certain auto antibodies present in the patient's serum may not be detected by ANA profile if it does not contain that specific antigenic substrate. Hence ANA -IFA is considered to be the gold standard screening test while ELISA profile is taken as the confirmatory test.[23]

The results of various studies which had compared ANA -IFA with ANA profile are inconsistent with regards to sensitivity, specificity and predictive values.[24-26] Some studies have shown moderate agreement between Enzyme immune assay and IFA.[15,24,26] While some have found that IFA is better others have shown that ELISA is superior.[20,22,27,28]

In the present study, we found that ANA IFA pattern correlated with the disease in 63%; ANA profile with the disease only in 49%; On cross tabulation, we observed that ANA testing by immunoprofile has a sensitivity of 65% with a positive predictive value of 69% when compared to standard IFA. Present study observation was similar to that noted by Priyadarshini et al, who compared ANA profile with standard IFA and reported ELISA has a sensitivity of 71.43% and specificity of 86.84% when compared with IFA12. There are studies which had reported that ELISA with improved profile is as good as IFA.

In a study by Kolahi et al, the sensitivity and specificity of immunoblot assay (ANA profile) in comparison with IF assay was 98.65% and 90.91% respectively.[17] Another study which had compared ANA detection by ELISA kit with IFA had reported that ELISA showed a reasonably good sensitivity (90.7%) and positive predictive value (89.1%) when compared to IFA method.[28] Raman et al, reported that sensitivity of LIA when compared to IFA was 89% and specificity was 42%.[19]

The present study also observed that IFA correlated well with the disease than ANA profile. Compared to standard IFA, the sensitivity and specificity of ANA profile in correlating with the disease was 46% each; positive predictive value of 59% and negative predictive value of 33%.

The present study is unique in that we assessed the results of ANA profile and IFA with respect to individual disease. We found that in SLE, ANA profile is as good as Immunofluorescence assay in picking up the disease with a sensitivity of 90%. Authors noted a high PPV of 82% in Sjogren's syndrome; 76% in MCTD by ANA profile when compared with IF method. All the scleroderma patients were picked up by ANA profile while IFA fails to detect the disease in one.

This observation tells that ANA profile is a very useful test in SLE and scleroderma. When the specific antibodies are positive in Sjogren's syndrome and MCTD, it is very likely that person is suffering from that specific autoimmune disease.

On correlation of ANA profile and IFA with the disease, we found that in SLE, ANA profile correlated well with the disease with a sensitivity of 80% and PPV of 44%. In Sjogren's syndrome, we found that ANA profile scored well in ruling in the disease with a high specificity of 86% and a very good PPV 80%. Authors also found a very good correlation of ANA profile with the disease in Scleroderma, with 100% sensitivity and NPV; Similarly, in MCTD, in 75% of the cases ANA profile is correct in predicting the disease.

Authors had used a mixed population of autoimmune diseases with small numbers which might again differ when used in larger sample.

CONCLUSION

ANA profile correlation with the disease is less when compared to Immunofluorescence assay. Also, ANA profile has a very low sensitivity and specificity when compared to IFA. ANA detection by immunofluorescence remains the investigation of choice when compared to ANA profile in the diagnosis of autoimmune disorders. In certain diseases like SLE and Scleroderma, ANA profile is as good as IF assay in picking up the disease and correlation with the disease.

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