



Research Article

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Role of Antinuclear Antibody Testing: ANA IIF with Immunoblot in Autoimmune Disorders in a Tertiary Care Hospital in India

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Abstract

Introduction: Antinuclear antibody detection by indirect immunofluorescence technique (ANA IIF) is valuable tool in screening for autoimmune disorders. ANA IIF is recommended screening test by American & European rheumatology society.

Material & Method: The retrospective study was conducted in tertiary care NABH accredited hospital in western India in the department of Pathology. Indirect immunofluorescence aSSA(RO60K)y was done on EUROIMMUN system that uses Human epitheloid cells (Hep 2) as substrate on slides. ANA immunoblot was performed on EUROIMMUNE IIFT Mosaic: Basic Profile 3A (EUROIMMUN Lubek, Germany) and Immunoblot were performed on EUROLINE ANA PROFILE 18 (EUROIMMUN Lubek, Germany).

Results: There were 3984 patients in the year 2025 that underwent ANA testing 443 (12.2%) had both ANA and Immuno blot done who are included in the study. ANA positivity was seen in 163 patients (4.5%). The most common age group was between 40-60 years with 79 (48.5%). Female preponderance was seen in study population- 138(84.7%) cases. In terms of intensity of ANA, majority of positive cases were of (+) intensity 88 (53.9%) followed by (++) 42 (25.8%), 32 (19.6%) and (+++++) 1 (0.7%). The most common pattern was Fine speckled – 87 (53.37%), followed by Cytoplasmic -33 (20.25%), Nucleolar – 17 (10.43%), Coarse speckled – 9 (5.4%), Homogenous – 5 (3%), Nuclear Dot -2 (1.2%).

Sensitivity of ANA testing in the present study was 99.14%, Specificity- 85.32%, Positive Predictive Value-70.55%, Negative Predictive Value-99.64% and Accuracy-88.94%. In terms of ANA pattern & antigen detection on immunoblot, the most common antigen detected was SSA(RO52) in 18% (35) cases, followed by RNP by 34.9% (18) & SSA (RO60K) in 14.4% (28) patients.

Discussion: The prevalence of autoimmune disorders detected by ANA IIF in the present study was 4.5%, findings are concordant with other literature studies. The only limitation of study was that samples included those patients where simultaneous ANA IFA and ANA immunoblot were ordered. As the samples are from single institution the findings of the study cannot be generalized.

Conclusion: The wide spread use of ANA IIF test and immunoblot mandates clinical correlation and in case ANA IIF negativity should not exclude diagnosis of autoimmune disorder.

Keywords: Antinuclear antibody testing, ANA IIF, ANA immunoblot, Autoimmune disorders

Introduction

Autoimmunity is aberrant immunity to self-antigens where the immune system undergoes deregulation in identifying self from non self-antigens [1]. This results in organ specific or non-organ specific disease [2]. In organ specific autoimmune disease single tissue or organ is targeted whereas in non-organ specific autoimmune disorder there is systemic involvement. The prevalence of autoimmune diseases is 3-5% in developing nations [3]. Generally, women are more commonly affected than men [4].

Antinuclear antibody detection by indirect immunofluorescence technique (ANA IIF) is valuable tool in screening for autoimmune disorders. ANA IIF is recommended screening test by American & European rheumatology society.

ANA testing refers to detection of antibodies directed against cell components – Anti DNA, anti RNA, anti-Histone & anti nucleosome. This autoantibody reaction results in an inflammatory response causing tissue damage and autoimmune disorders [5]. The sign and symptoms depend on the organ targeted by auto antibodies. The auto antibodies circulated in human serum are detected by immunofluorescence microscopy. ANA testing is a screening test and for exact identification of specific antigen immunoblot test is employed as ANA IIF cannot identify specific antigen.

There are limited studies from India on ANA testing and correlation with immunoblot for autoimmune disease. The present study is aimed to evaluate performance of immunofluorescence detection of auto antibodies and their correlation with immunoblot techniques.

Material & Method

The retrospective study was conducted in tertiary care NABH accredited hospital in western India in the department of Pathology. The data was anonymised for identity and was extracted from records, hence exempted from ethics committee review. The study

included all patients undergoing ANA & immunoblot testing in the year 2025 (Jan – Dec)

The blood samples were collected in plain red topped vial with separation of serum as per standard protocol.

Indirect immunofluorescence in EUROIMMUN system uses Human epithelial cells type 2(Hep 2) as substrate on slides which are considered as gold standard for ANA detection [3]. ANA IIF testing was done as per manufacturer instructions. In the first step samples were diluted with Phosphate buffer solution (PBS) in 1:100 ratio, then incubated with Hep 2 cells for 30 minutes, washed with PBS – Tween, incubated with addition of enzyme conjugate for 30 minutes and again washed with PBS-Tween. The slides were mounted and visualized under fluorescent microscope by two experts and reported ANA patterns as per international consensus guidelines [6]. The serum samples were diluted with PBS in a ratio 1:1000 followed by incubation with test strips containing appropriate antigens. The antigen – antibody reaction was visible on strips as dark purple bands, which were further scanned using Euroline ANA scanning software. ANA profile was performed on EUROIMMUNE IIFT Mosaic: Basic Profile 3A (EUROIMMUN Lubek, Germany) and Immunoblot were performed on EUROLINE ANA PROFILE 18 (EUROIMMUN Lubek, Germany).

Results

There were 3984 patients in the year 2025 that underwent ANA testing in the department, out of total patients undergoing ANA testing, 443 (12.2%) had both ANA and Immuno blot done who are included in the study. (Figure 1) ANA positivity was seen in 163 patients (4.5%) – (Table 1). The most common age group was between 40-60 years with 79 (48.5%) patients followed by 20-40-year age group – 41 (25.2%) & 35 (21.5%) were above 60 years of age. (Table 2) Female populations were predominant group comprising of 138(84.7%) cases (Table 3).

Table 1: ANA & immunoblot confirmation.

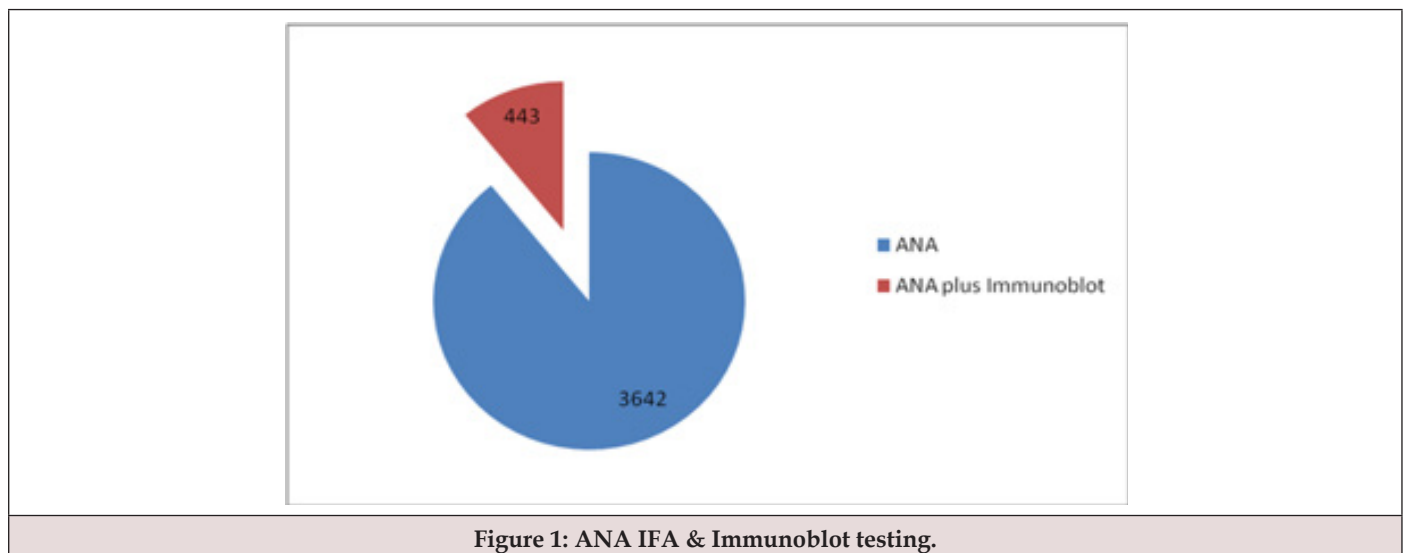
ANA	Total	
Negative	3461	95.50%
Positive	163	4.50%
Grand Total	3624	

Table 2: Age wise distribution of patients.

Age group	Total	
0-20	8	4.90%
20-40	41	25.20%
40-60	79	48.50%
>60	35	21.50%
Grand Total	163	

Table 3: Sex wise distribution of patients.

	Total	
Male	25	15.30%
Female	138	84.70%
Grand Total	163	



In terms of intensity of ANA, majority of positive cases were of (+) intensity 88 (53.9%) followed by (++) 42 (25.8%), 32 (19.6%) and (+++++) 1 (0.7%) – (Table 4). Table 5 shows pattern of distribution on ANA, the most common pattern was Fine speckled – 87 (53.37%), followed by Cytoplasmic – 33 (20.25%), Nucleolar

– 17 (10.43%), Coarse speckled – 9 (5.4%), Homogenous – 5 (3%), Nuclear Dot – 2 (1.2%). The rest of patterns were uncommon with single cases reported for centromere, cytoplasmic granular, fine coarse speckled and other mixed patterns.

Table 4: ANA intensity distribution.

ANA Intensity	Total	
+	88	53.90%
++	42	25.80%
+++	32	19.60%
++++	1	0.70%
Grand Total	163	

Table 5: ANA pattern distribution.

ANA Pattern	Total	
Fine Speckled	88	53.37%
Cytoplasmic	33	20.25%
Nucleolar	17	10.43%
Coarse Speckled	9	5.40%
Homogenous	5	3.00%
Nuclear Dot	2	1.20%

Mix (Fine Speckled and Cytoplasmic)	2	1.20%
Mix (Fine Speckled and Homogenous)	1	0.60%
Mix Pattern (Nucleolar & Homogenous)	1	0.60%
Spindle Fiber	1	0.60%
Centromere	1	0.60%
Cytoplasmic Granular	1	0.60%
Fine Coarse Speckled	1	0.60%
Mix (Coarse Speckled and Cytoplasmic)	1	0.60%
Mix (Cytoplasmic Fibrile and Fine Speckled)	1	0.60%
Grand Total	163	

In terms of ANA intensity and pattern observed (Table 6) the trend showed, Fine speckle pattern seen in 62.5% (55) patients, followed by Cytoplasmic pattern – 20.5% (18%), Nucleolar – 10.2% (9) & coarse speckle – 4.5% (4) cases for ANA intensity (+). Similarly, for (++) ANA intensity the most common pattern was Fine Speckle – 40.5% (17) patients followed by cytoplasmic pattern – 33.3% (14) cases. Fine speckle was most common pattern seen

in (+++) ANA intensity followed by homogenous pattern in 12.5% (4) patients. For (++++ ANA intensity there was a single case with coarse speckled pattern. Overall, the most common pattern was Fine speckle – 53.4% (84) followed by Cytoplasmic pattern – 20.2% (33), Nucleolar – 10.4% (17), coarse speckle – 5.5% (9) followed by other patterns.

Table 6: ANA Intensity & pattern.

ANA Intensity	C	CS	CY	FCS	FS	H	MCSCY	MCFFS	MFSH	MNUH	MFSCY	ND	NU	SF	Total
+		4 (4.5%)	18 (20.5%)		55 (62.5%)	1 (1.1%)						1 (1.1%)	9 (10.2%)		88 (100%)
++	1(2.4%)	2 (4.8%)	14 (33.3%)		17 (40.5%)							1 (2.4%)	6 (14.3%)	1 (2.4%)	42 (100%)
+++		2 (6.3%)	2 (6.3%)	1 (3.1%)	15 (46.9%)	4 (12.5%)	1 (3.1%)	1 (3.1%)	1 (3.1%)	1(3.1%)	2 (6.3%)		2 (6.3%)		32 (100%)
++++		1 (100%)													1 (100%)
Grand Total	1(0.6%)	9 (5.5%)	33 (20.2%)	1(0.6%)	87 (53.4%)	5 (3.1%)	1 (0.6%)	1 (0.6%)	1 (0.6%)	1(0.6%)	2 (1.2%)	2 (1.2%)	17 (10.4%)	1 (0.6%)	163 (100%)

Note*: Centromere- C, Coarse Speckle- CS, Cytoplasmic- CY, Fine Coarse Speckled- FCS, Fine Speckle- FS, Homogenous-H, Mix (Coarse Speckled and Cytoplasmic)- MCSCY, Mix (Cytoplasmic Fibrile and Fine Speckled)- MCFFS, Mix (Fine Speckled and Homogenous)-MFSH, Mix Pattern (Nucleolar & Homogenous)- MNUH, Mix(Fine Speckled and Cytoplasmic)- MFSCY, Nuclear Dot- ND, Nucleolar- NU, Spindle Fiber- SF.

Table 7 shows 2x2 table for ANA & immunoblot testing. Of the total 163 positive case on ANA 115 (70.56%) were positive on blot. There was only a single case where ANA was negative but blot was positive – False Negative. There were 48 cases (14.68%) of cases where immune blot was negative for ANA positivity – False positive

on ANA. Immunoblot the gold standard, Sensitivity of ANA testing in the present study was 99.14%, Specificity – 85.32%, Positive Predictive Value-70.55%, Negative Predictive Value-99.64% and Accuracy-88.94%.

Table 7: ANA – Blot (2 x 2) table.

ANA vs Blot	ANA Positive	ANA Negative	Grand Total
Immuno blot Positive	115	1	116
Immuno blot Negative	48	279	327
Grand Total	163	280	443

Table 8 shows the antigens associated with different ANA intensities with total 66.5% (35) cases. SSA(RO52) was the most common antigen in all ANA intensities.

Table 8: ANA intensity with associated antigen on immunoblot.

Statistics	Value	95% CI
Sensitivity	99.14%	95.29% to 99.98%
Specificity	85.32%	81.01% to 88.97%
Positive Predictive Value (*)	70.55%	64.84% to 75.69%
Negative Predictive Value (*)	99.64%	97.54% to 99.95%
Accuracy (*)	88.94%	85.64% to 91.7

Antigen	MI2	KU	RNP	SM	SSA (RO60K)	SSB	SSA (RO52)	SCL 70	PM100	JO1	CB	PCNA	DNA	NUC	HI	RIB	AMAM2	DFS70	
+	5 (6.4%)	8 (10.3%)	5 (6.4%)	5 (6.4%)	8 (10.3%)	4 (4.1%)	12 (15.4%)	5 (6.4%)	9 (11.5%)	3 (3.8%)	1 (1.3%)	3 (3.8%)	1 (1.3%)	2 (2.6%)	5 (6.4%)	0	2 (2.6%)	0	78 (100%)
++	1 (2.6%)	4 (10.3%)	4 (10.3%)	1 (2.6%)	4 (10.3%)	1 (2.6%)	6 (15.6%)	1 (2.6%)	3 (7.8%)	3 (7.8%)	2 (5.2%)	1 (2.6%)	0	2 (5.2%)	1 (2.6%)	0	3 (7.8%)	2(5.2%)	39 (100%)
+++	2 (2.8%)	2 (2.8%)	8 (11.1%)	3 (4.2%)	16 (22.2%)	4 (5.6%)	17 (23.6%)	1 (1.4%)	3 (4.2%)	2 (2.8%)	0	1 (1.4%)	2 (2.8%)	4 (5.6%)	5 (6.9%)	1 (1.4%)	0	1(1.4%)	72 (100%)
++++	0	0	1 (100%)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1 (100%)
Total	8 (4.2%)	14 (7.4%)	18 (34.2%)	9 (4.7%)	28 (14.7%)	9 (5.6%)	35 (66.5%)	7 (3.7%)	15 (7.9%)	8 (4.2%)	3 (1.9%)	5 (2.6%)	3 (1.6%)	8 (4.2%)	11 (5.8%)	1 (0.5%)	5 (2.6%)	3 (1.9%)	190 (100%)

In terms of ANA pattern & antigen detection on immunoblot, as shown in Table 9, the most common antigen detected was SSA(RO52) in 18% (35) cases, followed by RNP by 34.9% (18) & SSA(RO60K) in 14.4% (28) patients. SSA(RO52) antigen was most common antigen seen in 30 (24.4%) patients with fine speckled ANA pattern. (Table 10) In the cytoplasmic pattern most commonly associated antigen was JO1 seen in 5 (21.8%) cases. (Table 11) RNP

antigen was seen in 9 (64.3%) cases for coarse speckled pattern (Table 12) and PM – 100 was seen in 8 (80%) of ANA cases with nucleolar pattern. (Table 13) The most common antigen associated with homogenous pattern was DNA associated in 2 (50%) of cases. (Table 14) The nuclear dot & centromere pattern had CB antigen as most common antigen however the number of cases was very less in these patterns to draw a meaningful conclusion (Tables 15&16).

Table 9: ANA pattern with corresponding antigens on Immunoblot.

Pattern	Centromere	Coarse Speckled	Cytoplasmic	Fine Speckled	Homogenous	Mix (Coarse Speckled & Cytoplasmic)	Mix (Fine Speckled & Cytoplasmic)	Mix (Fine Speckled & Homogenous)	Mix Pattern (Nucleolar & Homogenous)	Nuclear Dot	Nucleolar	Total
MI2	0	0	1(4.8%)	6(4.9%)	0	0	0	1(12.5%)	0	0	0	8(4.1%)
KU	0	0	3(14.3%)	10(8.1%)	0	0	0	1(12.5%)	0	0	0	14 (7.2%)
RNP	0	9(64.3%)	1(4.8%)	6(4.9%)	0	1(20%)	0	1(12.5%)	0	0	0	18 (34.9%)
SM	0	3 (21.4%)	1(4.8%)	4(3.3%)	0	0	0	1(12.5%)	0	0	0	9 (4.63%)
SSA (RO60K)	0	0	0	26(32%)	0	1(20%)	1(25%)	0	0	0	0	28 (14.4%)

SSB	0	0	0	9(7.3%)	0	0	0	0	0	0	0	9 (4.6%)
SSA (RO52)	0	0	2(9.5%)	30(24.4%)	0	1(20%)	2(50%)	0	0	0	0	35 (18.0%)
SCL 70	0	0	0	5(4.1%)	0	0	0	0	1(50%)	0	1(10%)	7 (3.6%)
PM100	0	1(7.1%)	0	5(4.1%)	0	0	0	0	1(50%)	0	8(80%)	15 (7.7%)
JO1	0	0	5(21.8%)	2(1.6%)	0	0	1(25%)	0	0	0	0	8 (4.1%)
CB	1(100%)	0	0	1(0.8%)	0	0	0	0	0	1(50%)	0	3(1.5%)
PCNA	0	1(7.1%)	0	4(3.3%)	0	0	0	0	0	0	0	5 (2.6%)
DNA	0	0	0	0	2(50%)	0	0	1(12.5%)	0	0	0	3 (1.5%)
NUC	0	0	1(4.8%)	4(3.3%)	1(25%)	0	0	1(12.5%)	0	1(50%)	1(10%)	9(4.6%)
HI	0	0	0	8(6.5%)	1(25%)	1(20%)	0	1(12.5%)	0	0	0	11 (5.7%)
RIB	0	0	0	0	0	1(20%)	0	0	0	0	0	1 (0.5%)
AMA M2	0	0	4(19.0%)	1(0.8%)	0		0	0	0	0	0	5 (2.5%)
DFS70	0	0	0	2(1.6%)	0		0	1(12.5%)	0	0	0	3 (1.5%)
PL7		0	1(4.8%)	0	0	0	0	0	0	0		1 (0.5%)
LC1	0	0	1(4.8%)	0	0	0	0	0	0	0	0	1 (0.5%)
GP120	0	0	1(4.8%)	0	0	0	0	0	0	0	0	1 (0.5%)
Total	1(100%)	14 (100%)	21(100%)	123(100%)	4 (100%)	5 (100%)	4(100%)	8 (100%)	2(100%)	2(100%)	10(100%)	194(100%)

Table 10: Fine speckled pattern & corresponding antigens on immunoblot.

Pattern	Fine Speckled
MI2	6(4.9%)
KU	10(8.1%)
RNP	6(4.9%)
SM	4(3.3%)
SSA(RO60K)	26(21.1%)
SSB	9(7.3%)
SSA(RO52)	30(24.4%)
SCL 70	5(4.1%)
PM100	5(4.1%)
JO1	2(1.6%)
CB	1(0.8%)
PCNA	4(3.3%)
NUC	4(3.3%)
HI	8(6.5%)
AMA M2	1(0.8%)
DFS70	2(1.6%)
Total	123(100%)

Table 11: Cytoplasmic pattern & corresponding antigens on immunoblot.

Pattern	Cytoplasmic	Cytoplasmic & Coarse Speckled	Cytoplasmic & Fine Speckled
MI2	1(4.8%)	0	0
KU	3(14.3%)	0	0
RNP	1(4.8%)	1(20%)	0
SM	1(4.8%)	0	0
SSA(RO60K)	0	1(20%)	1(25%)
SSA(RO52)	2(9.5%)	1(20%)	2(50%)
JO1	5(21.8%)	0	1(25%)
NUC	1(4.8%)	0	0
HI	0	1(20%)	0
RIB	0	1(20%)	0
AMA M2	4(19.0%)	0	0
PL7	1(4.8%)	0	0
LC1	1(4.8%)	0	0
GP120	1(4.8%)	0	0
Total	21(100%)	5(100%)	4(100%)

Table 12: Coarse speckled pattern & corresponding antigens on immunoblot.

Pattern	Coarse Speckled
RNP	9(64.3%)
SM	3 (21.4%)
PM100	1(7.1%)
PCNA	1(7.1%)
Total	14(100%)

Table 13: Nucleolar pattern & corresponding antigens on immunoblot.

Pattern	Nucleolar
SCL 70	1(10%)
PM100	8(80%)
NUC	1(10%)
Total	10(100%)

Table 14: Homogenous pattern & corresponding antigens on immunoblot.

Pattern	Homogenous	Homogenous & Fine Speckled	Homogenous & Nucleolar
MI2	0	1(12.5%)	0
KU	0	1(12.5%)	0
RNP	0	1(12.5%)	0
SM	0	1(12.5%)	0
SCL 70	0	0	1(50%)
PM100	0	0	1(50%)
DNA	2(50%)	1(12.5%)	0
NUC	1(25%)	1(12.5%)	0

HI	1(25%)	1(12.5%)	0
DFS70	0	1(12.5%)	0
Total	4(100%)	8(100%)	2(100%)

Table 15: Nuclear dot pattern & corresponding antigens on immunoblot.

Pattern	Nuclear Dot
CB	1(50%)
NUC	1(50%)
Total	2(100%)

Table 16: Centromere pattern & corresponding antigens on immunoblot.

Pattern	Centromere
CB	1(100%)
Total	1(100%)

Discussion

The prevalence of autoimmune disorders detected by ANA IIF in the present study was 4.5%. The other Indian study by Gupta, *et al.* [3] showed prevalence of 33% and in Kruthika, *et al.* study it was 2.12% [7]. The other studies from different countries showed prevalence of autoimmune disorders as: Brazil – 23.5% [8], US-25% [9], Australia-28% [10] & China-5.9 % [11]. Peenet, *et al.* in their study reported prevalence of 23.5% [12], Minz *et al.* in their study 18.9% [13] & Sebastian, *et al.* 38.2% [14]. The variations in prevalence rates are attributed to sample size, cut off value of dilution used by different laboratories and ethnicity of population under study. ANA positivity is also seen in normal healthy population [8,15] and therefore interpretation of ANA positivity should be done in clinical context & never in isolation. The most common ANA pattern observed in the present study was fine speckled pattern associated with SSA(RO52) and SSA(RO60K) antigens. The second most common pattern was cytoplasmic with associated antigens – KU & AMAM2. Coarse speckled pattern was associated with RNP & Sm antigen. In contrast to other literature studies homogenous pattern was seen in 10% of patients, with ds DNA antigen as most common antigen association. The findings are similar to the study by Kruthika, *et al.* from India. [7] In another study from India by Reshmy, *et al.* speckled ANA pattern was commonest pattern among 902 positive ANA samples [16]. Homogenous pattern was observed as most common pattern in study from Indian subcontinent among 262 cases of ANA IFA positive cases. [17] The findings of present study correlate in literature with study by Jamil A Al Mughales, *et al.* who observed speckled ANA pattern in their study on SLE patients.[18] Contrary to our study, Wang, *et al.* [19] in their study had homogenous pattern in 42% of cases followed by mixed pattern in 23.7% and speckled pattern in 16.9% of patients.

The speckled pattern in literature is associated with specific antigens on immunoblot indicating particular autoimmune

disorder like Sm antigen in SLE, Scl-70 in mixed connective tissue disorder and SSA(RO60K)/SSB in Sjogren syndrome. [19] Homogenous pattern association was seen in SLE [20], Nucleolar pattern with Scl on immunoblot with Sjogren syndrome [21] and centromere pattern with progressive systemic sclerosis. [22] The speckled pattern is seen in various autoimmune disorders like Mixed connective disorders, scleroderma, SLE & Sjogren syndrome. Homogenous pattern seen in SLE patients. The corresponding antigens obtained in the present study correlate with specific autoimmune diseases.

Autoimmune diseases are more common in females in comparison to males due to estrogen that potentiates immune signalling resulting in autoimmune antibodies [23] and genetic factors like partial inactivation of X chromosome leading to increased expression of autoimmunity related antigens [24].

Sensitivity & specificity of ANA IFA in the present study were 99.14% & 85.32% respectively with diagnostic accuracy of 88.94%. Single case which was ANA negative turned out to be positive on ANA immunoblot showed CB antigen.

Conclusion

The prevalence of autoimmune disorders detected by ANA IIF in the present study was 4.5%. The most common pattern was Fine speckled pattern. SSA(RO52) was the most common antigen followed by SSA(RO60K). The wide spread use of ANA IIF test and immunoblot mandates clinical correlation and in case ANA IIF negativity should not exclude diagnosis of autoimmune disorder.

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