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Pattern and presentation of ANA by Immunofluorescence and Line Immuno Assay

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Abstract:

Background: Autoantibodies are the hallmark of autoimmunity, of which anti-nuclear antibody (ANA) is of key importance. ANA has the capability of binding and destroying certain structures within the nucleus of the cells. ANA are detected in the serum of patients suffering from autoimmune diseases. There is therefore a need to evaluate the pattern of ANA positivity in autoimmune diseases in Indian settings. **Methods:** This cross-sectional study was carried among 165 samples received with a suspicion of auto immune diseases. All the samples were positive for ANA with a dilution factor >1:100. Data regarding the clinical details were captured from the hospital records. The screening of serum samples for ANA was carried out by ImmunoFluorescence Assay. The confirmation of ds DNA was performed by Crithidia lucilae ImmunoFluorescence and confirmation of specific autoantibodies was done by Line Immuno Assay. **Results:** Majority of the participants were over 30 years of age (66.7%) and were females (80%). Majority of the positive samples were diagnostic of Systemic Autoimmune Rheumatic Disorders (SARD) (61.8%) of which majority were Systemic Lupus Erythematosis (53.9%) followed by Rheumatic arthritis (22.5%). The most common staining pattern seen was speckled nucleus (46%) followed by homogenous nucleus (22%). **Conclusion**: Clinical diagnosis obtained from the medical records should be evaluated with IFA and LIA results. The simultaneous utilisation of both these methods in a patient with strong suspicion of autoimmune disorders increases the sensitivity, provides much more information about the combination of results and facilitates the interpretation of patterns.

Keywords: Anti Nuclear Antibodies, Autoimmune diseases, Immunofluorescence assay, Line Immuno Assay, Systemic Lupus Erythematosis

Introduction

Autoimmune diseases are a family of chronic, disabling illnesses characterized by presence of autoantibodies resulting in loss of organ function. While many of these diseases are rare, collectively they affect millions of people globally and – for reasons unknown – their prevalence is rising. Auto immune diseases are lifetime diseases associated with increased morbidity,

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mortality, disability and poor quality of life. They also result in a considerable psychological and economic burden to both the individuals and family alike.[1]

Autoantibodies are the hallmark of autoimmunity, of which anti-nuclear antibody (ANA) have taken the centre stage for the past 60 years. ANA are a specific class of Autoantibodies that have the capability of binding and destroying certain structures within the nucleus of the cells. ANA are detected in the serum of patients suffering from autoimmune diseases. Detection of anti-cellular antibodies of the ANA family is pivotal to the diagnosis of many autoimmune diseases. [2]

Immunological assays for the detection of antinuclear antibodies (ANA) are useful and important complementary tools for the diagnosis and follow-up of patients with autoimmune diseases.ANA are usually screened by indirect immunofluorescence using HEp-2 cells, due to its great sensitivity. The different possible patterns, the intensity, and the titres can be observed by this test. [3]

The formation of ANA is indicative of several stages in a disease. ANA is used as a prognostic marker for several diseases. Lower amounts of these antibodies can be seen in the normal population as well, a spurt in titers is seen in patients with connective tissue diseases (CTD). Several physiological and pathological factors might favour the development of ANA even in the non-rheumatic population, such as pregnancy, advanced age, family history of autoimmune disease, as well as infectious, cardiovascular or oncological diseases. This situation conveys challenges such as interpretative standardization.[4]Their detection with high sensitivity and specificity is therefore of utmost importance.**[5]**

It is for the same reason that a close attention to the titres should be analysed in which the ANAs are reported, taking into account that in healthy individuals, antibodies should be negative or can be present in low titres. The use of these tests depends on the knowledge of their clinical classification criteria of each disorder in order to contribute to an appropriate diagnosis.

Though positive fluorescence staining indicates the presence of ANA, it does not, however, allow precise identification of these antibodies. For this purpose specialized techniques such as enzyme linked immunosorbant assay (ELISA), Western blotting or line immunoassay are employed. A broad spectrum of specific antibodies has been associated with each specific rheumatic disease entity as evidenced in several studies.**[1]** However, most of these have been carried out on Western population, but one needs to recall that immunity status, individual response to disease, type of antibodies, all, varies with ethnicity. There is therefore a need to evaluate the pattern of ANA positivity in autoimmune diseases in Indian settings.

Objectives

This study was carried out to evaluate the pattern and clinical significance of ANA positivity among patients with autoimmune diseases.

Methodology

Study setting

This cross sectional study was carried out in the Department of Microbiology of our tertiary care hospital for a period of one year between February 2015 and 2016.

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Study participants and selection criteria

All serum samples received in our laboratory for the purpose of screening of ANA antibodies were taken up for the study. Positive ANA samples with a dilution factor of < 1:100 was not included in the study. A total of 2320 serum sample of patients with suspicion of autoimmune disorders were screened for anti-nuclear antibodies by Indirect Immunofluorescence assay during the study period. Samples tested positive for ANA by IFA > 1:100 dilution were included in the study. A total of 165 samples were studied.

Ethical approval

Approval was obtained from the Institutional Ethics Committee prior to the commencement of the study. (CSP – MED/14/OCT/19/188).

Data collection

Data regarding the clinical history and participant demographics were captured using a structured proforma from the hospital records. The screening of serum samples for ANA was carried out by ImmunoFluorescence Assay (IFA). The confirmation of ds DNA was performed by Crithidia lucilae ImmunoFluorescence (CLIF) and confirmation of specific autoantibodies was done by Line Immuno Assay (LIA). All procedures were followed from the instructions given in EUROIMMUN kit insert and EUROLINE scan kit insert.

Screening of serum samples for ANA by Immunofluorescence assay (IFA)

Fluorescence pattern

- Positive reaction: The cell nuclei show a distinct fluorescence, which is characterized by certain patterns.
- Negative: The nuclei show no specific fluorescence.
- In each field evaluated, both interphase nuclei and mitotic cells were examined, in several areas if possible.
- If the positive control showed no specific fluorescence pattern or the negative control showed a clear specific fluorescence, the test was repeated.

Qualitative evaluation

- A titer of 1:100 or greater that results in a positive reaction was considered positive.

Calculation of results

The extinction value of the calibration for each individual antigen has to be multiplied by a lot and antigen specific factor. This provides the upper limit of the normal range (cut-off). The individual factors are stated on the included protocol with target values. Values above the recommended cut-off are to be considered as positive, those below as negative. The negative control serum functions as an internal control for the reliability of the test procedure and should be assayed with each test run. Besides this qualitative interpretation a gradual estimation of the result is possible by calculating a ratio according to the following formula:

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$$\frac{\text{Extinction of patient samples}}{\text{Cut-off extinction}} = \text{Ratio}$$

Confirmation of ds-DNA by immunofluorescence

The flagellate Crithidia lucillae possesses a giant mitochondrion containing dsDNA (Kinetoplast) that, in general, does not show nuclear antigens except for dsDNA. Antibodies reacting with the kinetoplast are only directed against dsDNA.

In the case of positive result, a distinct, homogenous,in parts circular fluorescence of the kinetoplast can be identified. Essentially the same pattern is found as for the positive control.

For negative samples, the kinetoplast shows no staining. A positive reaction at 1:10 is considered positive and indicative of SLE.

Detection of antibodies against nuclear antigens (IgG) by Line Immuno Assay (LIA)

Based on signal intensity, the results can be divided into negative and positive results. Results in the borderline range from 6-10 should be evaluated as increased but negative. An indirect immunofluorescence test should be performed in parallel with determination of cell nucleus antibodies by EUROLINE.

All the samples were further tested for a panel of 15 different antigens towards human antibodies of IgG class by qualitative Immunoblotting technique. These included Ribonucleoprotein (RNP)/ Smith antigen (Sm), Smith antigen alone, Ro-52, SS-A (Ro), SS-B (La), PCNA, Scl-70, PM-Scl, Jo-1 Ribonucleoprotein –P, ds DNA, Nucleosomes, Histones and AMA-2.

Results

This cross sectional study was carried out among 165 samples received in the Department of Microbiology over a period of one year. Majority of the participants were over 30 years of age (66.7%) and were females (80%). Majority of the positive samples were diagnostic of Systemic Autoimmune Rheumatic Disorders (SARD) (61.8%) of which majority were Systemic Lupus Erythematosis (53.9%) followed by Rheumatic arthritis (22.5%). (Table 1)

The most common staining pattern seen was speckled nucleus (46%) followed by homogenous nucleus (22%). Only two mitosis positive patterns were seen by IFA method. (Figure 1-3)

Among the speckled nucleus types, majority were coarse speckled (82.7%) followed by fine and dense fine speckled (8%). Among the cytoplasm staining, majority were non specific speckled (33.3%) followed by homogenous (29.2%). (Table 2)

Among the total samples tested, ANA positivity by LIA was present in 75.8% of the samples. Homogenous nucleus was present in 78 samples of which DS DNA was present in 32.1% of the

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samples. Speckled nucleus was present in 87 samples of which RO 52 was present in majority of them (32.2%). (Table 3)

Majority of the samples of MCTD, RA and SLE showed speckled pattern (65%, 65.2% and 50.9% respectively) while homogeneous pattern was highest in SLE (38.2%). Cytoplasm was highest in MCTD (35%) followed by RA (13.1%). (Table 4)

Among the samples showing speckled nucleus, 1:100 dilution showed + 1 positivity in 71.4% of the samples while1:1000 dilution showed +1 positivity in 66.7%. Among the samples showing homogenous nucleus, majority of the samples in 1:100 dilution showed +3 positivity (69.2%) and 1:320 dilutions showed +3positivity (80%). Among the samples showing cytoplasm positivity, majority of the samples in 1:100 dilution showed +1 positivity (63.2%). (Table 5)

Among the samples tested positive for ds DNA by CLIFA, 43.9% of the samples belonged to those with homogenous nucleus, followed by speckled nucleus (42.1%) and mixed nucleus pattern (14%). (Table 6, Figure 4)

Among the samples positive by LIA, majority of the samples belonged to speckled nucleus (44%) followed by homogenous nucleus (28.8%). Majority of the negative ANA pattern by LIA belonged to samples showing speckled nucleus (50%) followed by cytoplasm (35%). (Table 7)

Discussion

Antinuclear antibodies (ANA) are the hallmark of autoantibody production in autoimmune diseases. Autoimmune serology is an important tool in the diagnosis of autoimmune disorders. Screening for ANA is widely performed by IIF on HEp-2 cells and has the ability to detect multiple antigens simultaneously at the same time. The prevalence of positive ANA testing in this population was 7.11%.[6] in 2013 in Turkey demonstrated a 21.9 % positivity of ANA and.[7] in 2001 with 23.5% in in a 4 year study period. Whereas Indian studies have reported a wide variation in the positivity rate ranging from 2% to 77%.[8-10] The variability in the rate of positivity could be due to the difference in selection of patients with suspicion of autoimmune disorders, the various commercial kits used for detection of ANA and the time duration adopted for the and the dilution in which they were considered positive. As previously reported in various studies,[1] the female preponderance was 3.5 times more than in males. Majority of the patients were aged between 31-40 years. This was in concordance with a study done by[10]

The most common staining pattern was observed in the Nuclear region 71% (N=118) followed by cytoplasm -14% (N=23), nucleolar region -7% (N=12) and mitotic cells (N=2) 1%. The most prevalent fluorescence pattern was nucleus speckled 46% (N=75), followed by homogenous 22% (N=36), cytoplasm 14 %(N=23), nucleolar 7% (N=12) and centromere 4% (N= 4%). Another 6% showed mixed fluorescent pattern. Rare patterns like centrioles, spindle fibres, actin filaments, golgi bodies were detected in 4 different serum samples. The results were similar to studies done by [7] and [11]

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A total of 121 samples were tested for ds DNA by *Crithidia lucillae* Immunofluorescent (CLIF) assay. All the samples with Dense Fine Speckled pattern, fine speckled & pleomorphic pattern were negative for ds DNA. These findings were similar to the study done by[7] Antibody reactivity was identified in 76% of ANA positive samples. The most commonly identified specificity was anti-ds DNA antibodies in 26%. Anti extractable antinuclear antibodies (ENA) were identified in 54.3 % of the samples. The other antibodies including anti-CENP was noted in 4%, anti-nucleosomes (13.3%), anti- histones (8.4%), anti-ribososmal P protein (6%) anti-mitochondrial antibodies –AMA (1.2%) and anti-PM-Scl in 1.8%.

The clinical association for the positive ANA samples showed that 62% had systemic autoimmune rheumatic disorders (SARD), and another 38% were nonspecific with no clear cut autoimmune diagnosis. The most common SARD was Systemic Lupus Erythematosis (N=55) with nucleus speckled and homogenous pattern and cytoplasm homogenous staining. These samples showed a positive association with ds DNA, Smith antigen, nucleosomes and ribosomal P protein. Whereas Mixed Connective Tissue Disease (N=20) showed speckled pattern in nucleus and cytoplasm with strong reactivities with anti-ribonucleoprotein P, anti-SS-A, anti-SS-B and Ro-52 antibodies. Rheumatoid arthritis (N=23) showed most of the fluorescent patterns in the nucleus, nucleoli and cytoplasm. Systemic sclerosis (N=2) patients showed antibody reactivity to anti-Scl-70 (N=2) and anti –CENP (N=1) whereas Sjogrens syndrome (N=2) patients had anti-SS-A, anti-SS-B and anti-RO 52 reactivities in 2 samples. The other clinical diseases were PUO (N=18), polyarthritis (N=10), hypothyroidism (N=9), hepatitis (N=7) and primary biliary cirrhosis (N=3) and diabetes mellitus & myalgia (N=7 each).

Table 1: Demographic profile of the study participants:

S. No	Characteristics	Frequency (N=165)	Percentage
1	**Age (in years)**		
	<30	55	33.3
	>30	110	66.7
2	**Sex**		
	Female	132	80.0
	Male	33	20.0
3	**Postitive ANA samples**		
	SARD	102	61.8
	Others	63	38.2
4	**Systemic auto immune rheumatic disorders (n=102)**		
	Systemic lupus erythematosis	55	53.9
	Rheumatoid Arthiritis (RA)	23	22.5
	Sjogren’s syndrome	2	2.0

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	Systemic sclerosis	2	2.0
	Mixed connective tissue disorder (MCTD)	20	19.6
5	**Others (n=63)**		
	Thyroid disorders	9	14.7
	Hepatitis	7	11.5
	Primary biliary cirrhosis	3	4.9
	Polyarthritis	10	16.4
	Myalgia	7	11.5
	PUO	18	29.5
	DM	7	11.5

Figure 1: Patterns of ANA presentation in the samples by IFA:

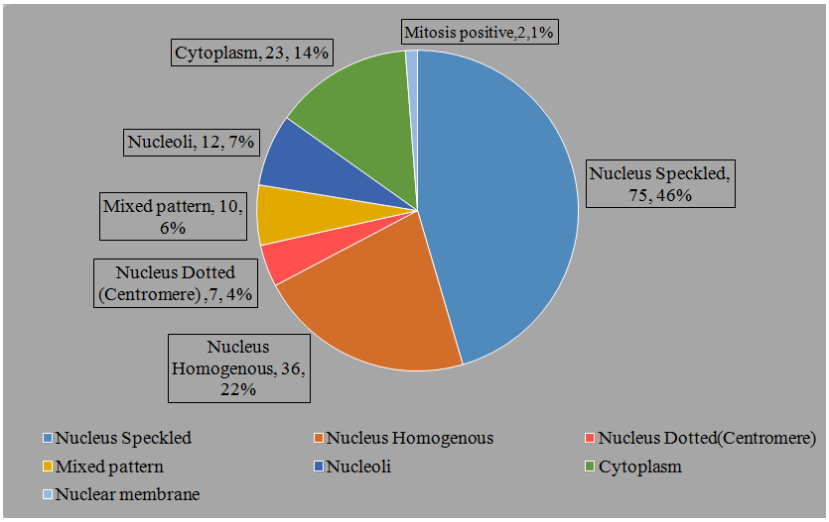

Table 2: Particulars related to nucleus and cytoplasm by IFA:

S. No	Characteristic	N	Frequency N (165)	Percentage (%)
1	**Details of nucleus speckled pattern**			
	Plemorphic	75	1	1.3
	Coarse speckled		62	82.7
	Fine speckled		6	8.0
	Dense fine speckled		6	8.0
2	**Cytoplasm staining pattern**			
	Homogenous	24	7	29.2
	Fine speckled		3	12.5
	Filamentous		2	8.3
	Coarse speckled AMA pattern		3	12.5

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	Speckled non specific		8	33.3
	Golgi bodies		1	4.2

Table 3: Pattern of ANA presentation by LIA:

S. No	Characteristics	N	Frequency	Percentage
1	**ANA positivity by LIA**			
	Negative	165	40	24.2
	Positive		125	75.8
2	**Nucleus homogenous**			
	DS DNA	78	25	32.1
	RNP/SM		15	19.2
	SS-A		15	19.2
	SCL-70		15	19.2
	Nucleosome		8	10.3
3	**Nucleus speckled**			
	RO 52	87	28	32.2
	RNP/SM		25	28.7
	DSDNA		10	11.5
	NUCLEOSOMEF		8	9.2
	HISTONE		8	9.2
	SS-A		4	4.6
	SS-B		4	4.6
4	**MIXED PATTERN**			
	NUCLEOSOME	34	6	17.6
	HISTONES		6	17.6
	DSDNA		4	11.8
	RNP/SM		4	11.8
	SS-A		4	11.8
	RO-52		4	11.8
	PM-SCL		3	8.8
	RIB-PRO		3	8.8
5	**Cytoplasm**			
	RIB.P PRO	17	7	41.2
	DSDNA		4	23.5
	AMA		4	23.5
	SS-A/SS-B		2	11.8

Table 4: ANA pattern in various diseases:

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S. No	Characteristics	N	Frequency	Percentage
1	**MCTD**			
	Speckled	20	13	65.0
	Cytoplasm		7	35.0
2	**RA**			
	Speckled	23	15	65.2
	Cytoplasm		3	13.1
	Homogenous		2	8.7
	Nucleoli		2	8.7
	Mixed		1	4.3
3	**ANA in SLE**			
	Speckled	55	28	50.9
	Homogenous		21	38.2
	Mixed		4	7.3
	Cytoplasm		2	3.6

Table 5: ANA- Dilution and intensity of fluorescence in various patterns

S. No	Characteristic	N	+1 N (42)	+2 N (13)	+3 N (20)	Total N(75) %
1	**Nucleus speckled *75**					
	1;100	75	15 (71.4)	1(4.8)	5(23.8)	21 (28.0)
	1;1000		10 (66.7)	3(20.0)	2 (13.3)	15 (20.0)
	1;320		17 (43.5)	9(23.1)	13(33.4)	39 (52.0)
2	**Nucleus homogenous*36**					
	1;100	36	2 (15.4)	2(15.4)	9(69.2)	13(36.1)
	1;1000		2 (25.0)	3(37.5)	3(37.5)	8 (22.2)
	1;320		0 (0.0)	2(20.0)	8(80.0)	10 (27.8)
	1;3200		0 (0.0)	2(40.0)	3(60.0)	5 (13.9)
3	**Mixed pattern*10**					
	1;100	10	1 (16.7)	4(66.6)	1(16.7)	6 (60.0)
	1;320		1 (25.0)	2(50.0)	1(25.0)	4 (40.0)
4	**Dotted pattern*7**					
	1;100	7	3(60.0)	2(40.0)	0(0.0)	5 (71.4)
	1;320		0(0.0)	1(50.0)	1(50.0)	2 (28.6)
5	**Nucleoli pattern*12**					
	1;100	12	1(16.7)	2(33.3)	3(50.0)	6(50.0)

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	1;320		3(50.0)	2(33.3)	1(16.7)	6 (50.0)
6	**Cytoplasm pattern*23**					
	1;100	23	12(63.2)	6(31.5)	1(5.3)	19 (82.6)
	1;320		0(0.0)	1(100.0)	0 (0.0)	1 (4.3)
	1:3200		3(100.0)	0 (0.0)	0 (0.0)	3 (13.1)

Table 6: Detection of dsDNA by Crithidia Lucillae IFA

S. No	Characteristic	ds DNA				Total N(121)
		Positive N(57)	%	Negative N (64)	%	
1	Nucleus homogenous	25	(43.9)	11	(17.2)	36 (29.8)
2	Nucleus speckled	24	(42.1)	51	(79.7)	75 (61.9)
3	Mixed nucleus pattern	8	(14.0)	2	(3.1)	10 (8.3)

Table 7: Confirmation of specific auto antibodies by line immune assay

S. No	Characteristic	ANA pattern				Total N(165)
		Positive N(125)	%	Negative N (40)	%	
1	Nucleus homogenous	36	(28.8)	0	(0.0)	36 (21.8)
2	Nucleus speckled	55	(44.0)	20	(50.0)	75 (45.5)
3	Centromere (nucleus dotted)	7	(5.6)	0	(0.0)	7 (4.2)
4	Nucleoli	10	(8.0)	2	(5.0)	12 (7.3)
5	Cytoplasm	9	(7.2)	14	(35.0)	23 (13.9)
6	Mixed	8	(6.4)	2	(5.0)	10 (6.1)
7	Mitosis	0	(0.0)	2	(5.0)	2 (1.2)

Figure -2: Nucleus homogenous pattern

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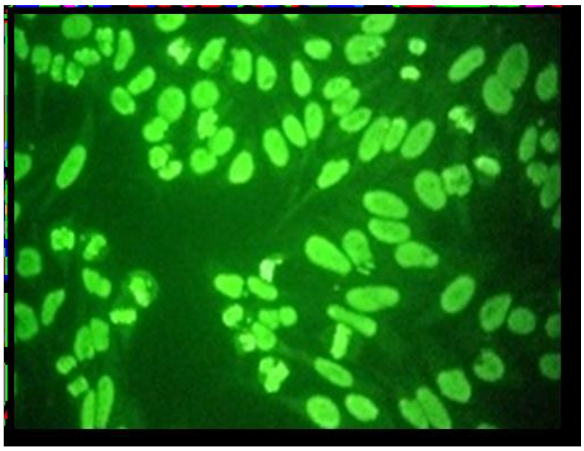


Figure 3: Nucleus coarse speckled pattern

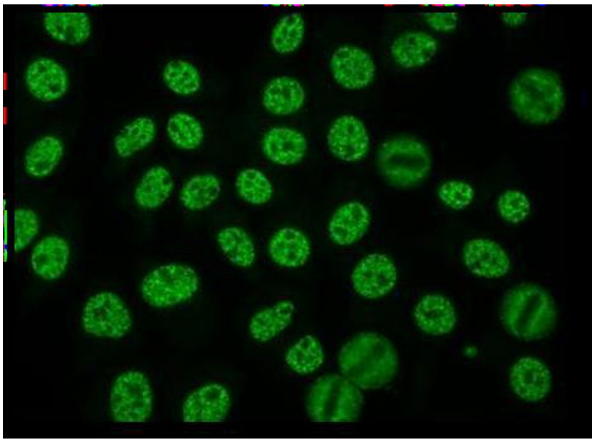
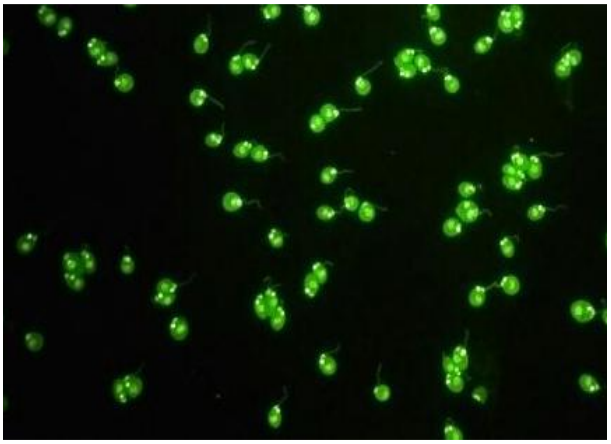


Figure-4: Positive DS DNA by Crithidia lucillae by IFA



Conclusion

Autoantibody tests have been used extensively in diagnosis and follow-up of patients in Rheumatology clinics. Immunofluorescent antinuclear antibody test using HEp-2 cells is still considered the gold standard for screening of autoantibodies. Among the many autoantibody specificities described, some have been established as clinically useful diagnostic markers and are

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included in the classification criteria of diseases. Clinical diagnosis obtained from the medical records should be evaluated with IFA and LIA results. The simultaneous utilisation of both these methods in a patient with strong suspicion of autoimmune disorders increases the sensitivity, provides much more information about the combination of results and facilitates the interpretation of patterns. There should also be a strong collaboration of clinics and laboratory in order to achieve an accurate diagnosis and avoid unnecessary treatment or failure of the same.

Limitations

Although the ANA patterns are helpful for narrowing down the tests for specific autoantibodies when performed and interpreted correctly, several potential pitfalls should be noted. First, interpretation is somewhat subjective, and weaker staining may not be reported. The reporting is subject to intra and inter observer variations. Second, the report on the staining pattern may not always accurately reflect the known cell biological location of the target antigen. In addition, it is important for the clinicians to interpret false positive results with caution.

Declaration

Conflict of interest – nil

Funding – nil

Ethical approval – obtained

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