Research Article

International Consensus on ANA Patterns (ICAP) classification tree revisited: A single centre report on four nuclear patterns from a tertiary care centre in India

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Abstract

Background: ICAP describes ANA patterns from AC- 0 to AC-29. They are further marked for competent or expertise reporting depending on ease of identification. There are some debatable patterns in ICAP which share similar features with a few others yet have a distinct identity and few others which are not addressed by ICAP but are described by BCA like Quasi-homogenous. This study analysed four nuclear patterns with overlapping features, namely Homogenous, speckled, Dense Fine Speckled70(DFS70) and quasi-homogenous to identify challenges posed in their identification due to overlapping features.

Methods: All samples which were reported as positive for the above four nuclear patterns (n=388) by IIF using HEp-2 cell were included in the study. LIA was performed on 103 such samples to look for association between the ANA patterns and specific antibody detected by LIA.

Results: DFS70 pattern is a rare pattern and existed in combination with other autoantibodies thus making its identification difficult on IIF. Homogenous pattern corresponded to AC- 29 (anti-topoisomerase, anti-Scl 70) which was probably due to wrong identification. Mixed pattern i.e speckled and homogenous was associated with Sm and U1sn RNP antibodies.

Conclusions: DFS 70 is a pattern with overlapping features of both homogenous and speckled and calls for expertise reporting. More awareness is required about AC 29 pattern as it is an overlap of five different components. Its identification poses significant challenges and is rightly placed in the expert reporting by ICAP. Mixed pattern (speckled and homogenous) referred to as Quasihomogenous by BCA needs to be addressed by ICAP.

Introduction

Antinuclear antibodies (ANA) are directed against nuclear self-antigens and are the hall mark of systemic autoimmune rheumatic diseases (SARD). The prevalence of autoimmune diseases is 3%–5% in developing countries with significant female preponderance [1]. A significant increase has been observed in overall incidence and range of autoimmune diseases after COVID -19 infection [2]. Many methods are available for detection of ANA of which indirect immunofluorescence (IIF) using Human epithelial cancer (HEp-2) cell line is considered to be the gold standard. IIF is the most commonly used screening tool on suspicion of SARDs or connective tissue diseases (CTDs) [3]. Different patterns are visualised under the fluorescent microscope which correspond to the different nuclear, cytoplasmic or mitotic antigens targeted by autoantibodies. Each pattern hence obtained gives vital clue to the probable antigen targeted and the clinical disease associated. A recent classification of the International consensus on ANA patterns (ICAP) based on HEp-2 patterns in the diagnosis of ANA-associated autoimmune diseases intends to achieve harmonisation in ANA reporting across the world [4]. This classification generates 29 different types of fluorescence patterns numbered from AC 1 to AC 29 and a negative pattern numbered AC 0. The patterns are distributed into 3 groups: nuclear, cytoplasmic, and mitotic. Each of these patterns is expected to reflect some clinical relevance. The disease associations may be confirmed further by specific tests like Enzyme linked immunoassay (ELISA), Line-immunoassay (LIA), chemiluminescence immunoassay (CLIA), FEIA (Fluorescent enzyme immunoassay) etc. to identify the antigen targeted. However not all anti-nuclear antibodies are associated with disease [5]. One pattern believed to be associated with apparently healthy individuals is the dense fine speckled pattern (AC-2), but this association only holds if the targeted antigen is confirmed as monospecific for DFS70.As per literature, DFS 70 is associated with non- autoimmune conditions [6]. At a titre of 1:40 serum dilution, 25–30% of healthy individuals may show ANA positivity, which increases with age [7]. In contrast to ICAP, The Brazilian Consensus on Autoantibodies (BCA) recognizes 34 different positive staining patterns observed by IIFT on HEp-2 cells. BCA proposed the distribution of these patterns into 5 groups: nuclear, cytoplasmic, nucleolar, mitotic, and complex. Nucleolar staining is a separate entity in the BCA which is included in the nuclear group in the ICAP classification and the complex patterns (CPs) which are not addressed by the ICAP classification are included in BCA [8]. The terms “mixed pattern” or “composite pattern” were introduced to describe those cases where more than one cellular component is targeted by a single antibody in the same sample or an overlap of patterns is observed due to the presence of more than one antibody [8]. The nuclear pattern as a whole is invariably the most common pattern reported in all studies worldwide [9]. Amongst this, the homogenous and speckled patterns are the most frequently reported. The dense fine speckled (AC-2) and anti-topoisomerase I (AC-29) patterns were previously often considered homogeneous, speckled or even mixed patterns due to overlapping features and the distinction till date is difficult at competent level of reporting [4]. This study was undertaken to understand the demographic profile, clinical manifestations and laboratory findings associated with these four similar yet different nuclear patterns, namely homogenous, speckled, DFS-70 and mixed (homogenous and speckled) in a tertiary health care set up in northern India.

Material and Methods

This is an analytical cross sectional study that was carried out in Biochemistry Central lab, AIIMS Patna in collaboration with departments of General Medicine, Dermatology, Paediatrics and Orthopaedics. The study was carried out after obtaining Institute ethical clearance from October 2023 to February 2024 with reference to letter number Ref.No.AIIMS/Pat/IEC/2023/1150. All samples for which an ANA screening by IIF (HEp-2) was advised by the treating clinician and subsequently processed were included in the study. All the samples which yielded a positive ANA screening report reporting four nuclear patterns, namely, homogenous (AC-1), speckled (AC-4 and AC-5), dense fine speckled ((AC-2) as per ICAP classification and mixed [homogenous and speckled] or quasi homogenous as per BCA classification(BCA3), were included as the study group. A total of 2294 samples were received for ANA screening by IIF HEp-2 during the study period. A total of 388 (16%) samples were positive for the four nuclear patterns committed in the study. The patients were tracked using Electronic medical records using Hospital information system for further details. Relevant history was taken by administering drafted questionnaire after obtaining informed consent. Data was collected and entered in MS excel.

Samples were processed using kits of Euroimmun (Lubeck, Germany), IIFT Mosaic HEp 20-10/Liver(Monkey) based on indirect immunofluorescence technique. The indirect immunofluorescence (IIF) test is considered gold standard for the determination of antibodies against nuclear antigens including cytoplasmic and mitotic components. Antibodies against cell nuclei can be determined on numerous substrates. The BIOCHIP technology facilitates different substrates to be combined in one test field (multiplex test) and incubated with individual patient serum. The substrate combination HEp-2 or HEp-2010 cells with primate liver allows dual confirmation of few patterns in a single approach. Combinations of HEp-2 cells and primate liver as substrate are incubated with diluted patient sample(1:100). Specific antibodies of classes IgA, IgG and IgM attach to the antigens if a positive reaction is obtained. The attached antibodies are stained with fluorescein labelled anti-human antibodies in a second step and visualised with the fluorescence microscope by Euroimmun (EUROSTAR) by two readers. The four ANA patterns were identified as follows keeping in mind ICAP guidelines and our past experience:

A. Homogenous pattern: The AC-1 pattern is characterized by a homogeneous nucleoplasm during interphase with an intensely

stained chromatin mass in a homogeneous hyaline fashion in mitotic cells. Primate Liver is positive for homogenous pattern and is associated with autoantibodies to double stranded DNA (dsDNA), nucleosomes, and histones, which are mostly related to Systemic Lupus Erythematosus (SLE).

B. Dense Fine speckled [DFS70]: The AC-2 pattern is characterized by a heterogeneity in the size and brightness of speckles in the nucleoplasm during interphase with a heterogeneously speckled chromatin in the metaphase plate. It is associated with autoantibodies against the dense fine speckled protein of 70 kD (DFS70) also known as lens epithelium-derived growth factor protein of 75 kD i.e LEDGF/p75.Primate liver is largely negative.

C. Speckled pattern: The AC-4(Fine speckled) and AC-5 (coarse speckled) are identified by fine, tiny and coarse speckles across the nucleoplasm respectively. Nucleoli is not stained. Mitotic cells do not reveal any staining of chromatin mass. This pattern corresponds to antibody against antigens U1sn RNP (uridine 1 small nuclear ribonuclear protein),SSA-Ro/SSB/La(Sjogren syndrome), Sm (smith antigen)etc.. Primate liver shows positive reaction.

D. Mixed pattern (homogenous and speckled): It has characteristics of both the speckled and homogenous pattern with nucleus of primate liver also showing positive reactivity.

E. Anti-Topoisomerase I(AC29): This pattern is characterized by five key elements: (1) prominent nuclear compact fine speckled pattern in interphase cells, (2) consistent strong fine speckled staining of condensed chromatin in mitotic cells, (3) strong staining of nucleolar organizing region (NOR) associated with condensed chromosomes in mitotic cells, (4)weak and delicate cytoplasmic weblike staining radiating from the perinuclear area to the plasma membrane, and (5) inconsistent staining of the nucleoli. Additionally, the Topoisomerase I-like pattern exhibits a subtle hazy interface between the nuclear fine speckled staining and the cytoplasmic staining. Unlike typical nuclear patterns with sharp borders, the Toposiomerase I nuclear staining displays a blurry border where the fine speckling in the nucleus extends into the adjacent cytosolic region to nucleus of hepatocytes. This pattern corresponds to antibody against antigen Scleroderma -70(Scl-70).

As a confirmatory testing for specific antigens, further line immunoassay (LIA) testing was performed using kits developed by Human diagnostics (Germany). The instrument used was fully automated HUMABLOT 44FA(Germany). LIA is a qualitative test which reveals reactivity of antibody to antigens coated as distinct lines on a membrane. A total of 18 antigens were capable of being detected in a single approach by ANA profile kit based on LIA(IMTEC-ANA-LIA-XL). Specific nuclear antigens are applied to nitrocellulose strips at equal distances which are then placed on respective rows of the incubation tray. A buffer containing blocking protein is added to rehydrate and to block free binding sites on the strips against unspecific binding. The membrane strips are incubated with prediluted serum samples (1:100) after discarding the blocking buffer. Autoantibodies present in the patient's sample bind to the antigens according to their specificity and are traced by alkaline phosphatase conjugated anti-human-IgG antibodies that appear as blue stained bands on the strips. The nuclear and associated cytosolic antigens applied as thin bands on a nitrocellulose membrane in our study included 18 antigens namely, dsDNA, Nucleosome, histone, SmD1, proliferating cell nuclear antigen(PCNA), Po(RPP-ribosomal P Protein), SS-A/Ro60, SS-B/Ro52, SS-B/La(Sjogren syndrome), CENP-B(centromere protein B), Scl-70, U1-snRNP, AMA-M2(anti-mitochondrial antibody), Jo-1, PM-Scl(polymyositis-Scleroderma), Mi-2, Ku and DFS 70. Qualitative measurement of IgG class of antibodies found in human serum against these antigens helps in diagnosis of a wide number of diseases which constitute SARDs. Patient's result were categorised age and sex wise. The results were graded as negative (-), equivocal (0), (+), (++), (+++) depending on intensity of band with reference to cut off control. The test result is negative if no band is recognised or if the band exhibits a smaller intensity in comparison to the cut -off control, equivocal if the intensity of the band and the intensity of the cut off control do not significantly differ and positive if a band exhibits a stronger staining in comparison to the cut-off control. Functional cut off serves the purpose of quality control.

Statistical Analysis

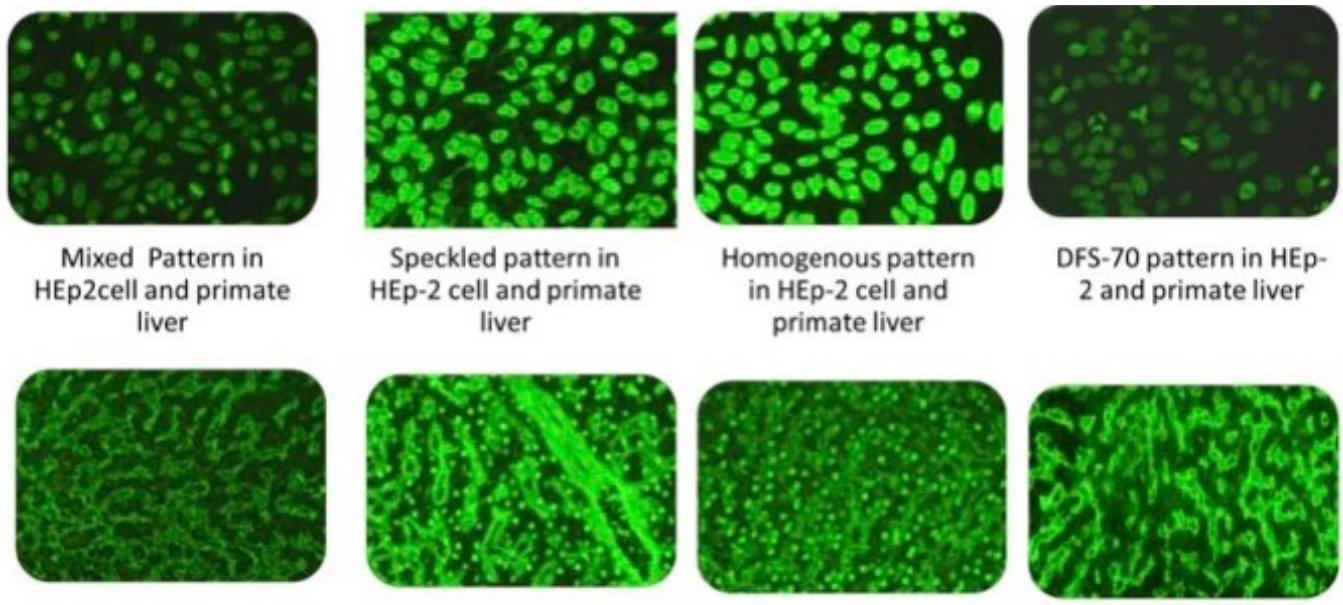
Descriptive statistics, such as means, standard deviations and ranges for continuous variables and frequencies and percentages for categorical variables was conducted to outline the baseline characteristics of the sample. The primary analysis involved chi square test to explore the association between categorical variables especially different ANA patterns (homogenous, speckled, DFS 70 and mixed: quasi-homogenous) and the antigens identified. All analyses were conducted using JAMOVI 2.3.28 [10,11].

Observation and Results

A total of 2294 samples were received for ANA screening.528 samples (23%) were from the IPD and 1766 (76.98%) samples were from the OPD. A total of 388 samples (16%) were reported as positive for the four nuclear patters included in our study.444 samples (19.3%) tested positive for other ANA patterns excluding the four described here. The total positivity rate was 35.3% including all the other ANA patterns. 105 (27.1%) and 283(72.9%) patients from IPD and OPD respectively tested positive for the four nuclear patterns. A total of 294(75.8%) females and 94(24.2%) males were positive for the nuclear patterns included in our study. The maximum number of patients (142) with a positive nuclear pattern belonged to the 21-40 age group amounting to 36.6% of the total patients. The medical specialities accounted for 301(77.6%) positive four nuclear

ANA patterns included in our study whereas 87(22.4%) positive nuclear ANA patterns were from surgical specialities. Amongst the specialities, General Medicine Department accounted for 42% of the positive nuclear samples included in our study, orthopaedic department accounted for 18.3% followed by pulmonary department at 10.6%, gastroenterology at 8.8%, paediatrics at 6.2% and dermatology at 5.2% followed by the other departments amounting for the remaining 9.0%.The most common pattern reported as per our study was the speckled pattern(69.8%) followed by homogenous(14.2%),mixed (13.9%) and the least common pattern reported was DFS 70 at 2.1%. The most common symptom reported was joint involvement (63.7%) followed by fever (51.8%) and fatigue (46.9%). Amongst the laboratory parameters, the most common finding reported was anaemia (60.3%). A total of 103 samples were also subjected to a confirmatory testing for ANA for specific antigen detection by LIA. As this was a non-funded study, samples which were advised for further testing by LIA by the treating clinicians were included(n=103) excluding the rest (n=285). The most common antibody observed on LIA in 103 patients was against U1snRNP (24.27%) followed by Ku (18.44%) and SSA-Ro60 (16.5%). Only 2 persons amongst 103 had antibody against Mi-2. No antibody was recorded against CENP-B and Jo1. We also looked for any association between ANA screening patterns and specific autoantibodies detected by LIA. Significant association was noted between mixed pattern and U1 snRNP(p=0.048) and Sm D1(p=0.042). Significant association was noted between homogenous pattern and dsDNA(p=0.024) and Scl 70(p=0.008). No significant association was noted between speckled pattern and any of the antigens detected on ANA profile by LIA. DFS70 pattern was not taken into account because of a small sample size(n=2) for which both ANA screening and corresponding reports by LIA were available.100% negative association was noted between the four nuclear patterns included in our study and CENP-B and Jo-1. Figure 1 shows the four different positive nuclear patterns obtained in our study (a) homogenous(b) speckled (c) mixed (homogenous and speckled) and (d) dense fine speckled (DFS-70).

Figure 1: Depicts Mixed,speckled,homogenous and DFS -70 patterns on HEp-2 cell line and Primate liver by IIF.



Discussion

Autoimmune testing is an important diagnostic aspect in a clinical setup. ANA screening is one of the most frequently performed tests as a first line investigation in a suspected case of SARD. HEp-2 cell line serves as an efficient substrate for the ANA test. However, it significantly increases the sensitivity, which often leads to a high-false positive rate. A biochip that incorporated primate liver along with the HEp-2 cells was introduced to further improve the performance of HEp-2 cell line especially in cases of ambiguity. Further reflex testing can be performed by commonly available techniques like ELISA, LIA etc to confirm the targeted antigen. Our study was performed to identify and evaluate the four similar yet distinct nuclear patterns, namely homogenous (AC-1), speckled (AC-4 and AC-5), dense fine speckled -70(AC-2) and mixed pattern (homogenous and speckled together also known as Quasi homogenous by BCA for ANA patterns) from a tertiary health care in a northern state of India, Bihar. Such a study to address the two debatable patterns on ANA screening, namely DFS-70 and Quasi Homogenous is probably the first of its kind in India.

The findings revealed many similarities with previously published studies. Sixteen percent (16.3%) positivity was reported for the four nuclear patterns on ANA screening in a time frame spanning 5 months. The total positivity rate was 35.3% including all the other ANA patterns. As the study was not population based, prevalence could not be estimated but a rough

calculation of disease burden could be proposed. The patterns included all grades of intensity reported. Few other studies from India in past have reported a prevalence of 38.2% (Sebastian et al, n=5066) and 18.9% (Minz et al, n=650) ANA positivity based on HEp-2 screening which included all ANA patterns reported in the study [12,13].A study conducted by Guo et al in China was a population based study on a sample size of 20970 people and the total positivity rate was 5.9% [14]. Female population was most commonly affected accounting for 75% of the total positivity in our study. Female predominance in the field of autoimmunity is already established. Beeson et al attributed estrogen as a potential modulator of autoimmunity [15]. Hayter et al proposed microchimerism, implication of X-chromosome encoded genes and random inactivation phenomena as the potential causes for female predominance amongst autoimmune diseases [16]. The most common age group afflicted in our population was 21-40 years accounting for 36.6% of the total positives for nuclear pattern. Table 1 depicts age and sex distribution of four different nuclear patterns in our study. Our study is in accordance with the findings of Beeson et al who has reported that autoimmune disorders are high in the 20 to 50 age group, while Hayter et al observed that the 20 to 29 age group presented with the highest prevalence of autoimmune disorders [15,16]. Cataudella et al have reported the highest prevalence of autoimmune diseases in the reproductive age group [17]. ANA positivity was maximum in the 30 to 39 age group patient population with a mean age of 37 ± 18 years in a study by Gupta et al from central India[9]. In a study from central India authors have emphasised that childbearing may be accountable for initial antigen stimulation or breach in tolerance to self-antigens contributing to the event of autoimmunity [9].

Table 1: depicting age and sex distribution of pour nuclear patterns in our study.

Sex wise distribution			
Age (years)	**Total number of females positive**	**Total number of males positive**	**Total**
0-20	52	17	69 (17.7%)
21-40	107	35	142 (36.6%)
41-60	108	22	130 (33.5%)
61-80	27	20	47 (12.1%)
Total	294	94	388

Screening tests that have high false positive rate have many undesirable consequences. When requested minus appropriate clinical rationale, tests that screen for rare diseases but have a high rate of false positivity can lead to multiple problems including misdiagnosis and potentially harmful follow-up testing and even inappropriate treatment. ANA is such a test with a very low positive predictive value. Out of a total of 2294 samples received for ANA testing by HEp-2 cell line ,388 were positive for ANA for the four nuclear patterns amounting to a positivity of 16.3%. Out of 819(35.7%) requisitions raised by the department of General Medicine at our hospital,163 were positive for the four nuclear patterns amounting to 19.9% positivity. In a study by Banhuk et al, 14 (8.1%) ANA test was advised by rheumatologists and 158 (91.9%) by physicians from other specialties. Amongst the positive results for ANA, 16.7% were advised by rheumatologists and the rest by other departments, whereas in the negative group, rheumatologists requested only 6.2% of the tests [18] implying that rheumatologists were more trained to evaluate and identify rheumatological diseases. The chances of ANA result being positive in a case suspected of a rheumatological disease by a rheumatologist was high as compared to other specialities. In a study in Korea, ANA positivity rate was 14.4% and varied according to the requesting department, with the highest rate for rheumatology (19.9%). ANA associated rheumatic disease (AARD) was diagnosed in 645 (0.69%) among all ANA tested patients. The diagnosis rate varied according to the requesting department with the highest for rheumatology and hemato-oncology (1.73% and 1.23% respectively). However, diagnosis of SARD was made in less than 1% of ANA tested subjects for all other departments. AARD was diagnosed in 4.74% among all ANA positive patients. SARD diagnosis rate among ANA positive patients was the highest for rheumatology, followed by nephrology and hemato-oncology (8.7%, 6.95%, and 6.86%, respectively). SARD diagnosis rate was the lowest among both ANA tested and ANA positive patients when requested by orthopaedics (0.14% and 1.23%, respectively). However, AARD diagnosis was made in only 1.73% of ANA tested patients even when it was requested from rheumatology thereby questioning the predictive value [19]. Patients with the highest pretest probabilities for AARD, as associated with the initial presenting symptoms are most likely referred to rheumatology. However even in those patients with a positive ANA test, the diagnosis of a rheumatic disease being made was very less. As we did not have rheumatology department, the maximum number of patients with fever and musculoskeletal complaints were attended to by General Medicine department. Hence, the maximum number of ANA positivity for nuclear samples (42%) was reported from the department of General Medicine followed by the department of Orthopaedics. Table 2 depicts the percentage positivity of samples from different departments for ANA screening test using IIF.

Table 2: depicting positivity rate from different Departments.

Department	Total number of requisitions raised on HIS[OPD]	Total number of requisitions raised on HIS[IPD]	Total number of requisitions raised inclusive of IPD and OPD	Total number of samples positive for four nuclear patterns included in our study	Positivity [%]
Dermatology	60	24	84(3.6%)	20	5.15
Gastroenterology	201	26	227(9.8%)	34	8.76
General Medicine	540	279	819(35.7%)	163	42.01
Orthopaedics	526	4	530(23.1%)	71	18.3
Paediatrics	66	84	150(6.5%)	24	6.19
Pulmonary Medicine	152	42	194(8.45%)	41	10.57
Others	221	69	290(12.6%)	35	9.02
Total	1766	528	2294	388	100

Amongst the four common nuclear pattern the most common pattern noted was speckled (69.8%), a finding which is very much in agreement with previous studies. In a study by Ramachandran et al in India, the most common pattern reported was speckled (52.9%) followed by homogenous (27.5%) in a study involving 204 SLE patients [20]. In alignment with our findings, a research from Saudi Arabia found speckled pattern (79.5%) as the most frequently reported followed by the homogeneous pattern (11.4%) [21]. DFS -70 pattern is a very rarely reported pattern and its frequency across world- wide studies have varied from 0.3% to 27% [22]. DFS 70 accounted for 2.1% of the total of four nuclear patterns in our study. The DFS 70 pattern is frequently observed in people without autoimmune diseases and with a positive ANA test [22]. DFS 70 is also known to have a positive association with diseases with an autoimmune basis like Raynaud's disease and idiopathic fibrotic alveolitis [23]. However, some studies have observed that patients with DFS 70 pattern were diagnosed with rheumatic autoimmune diseases [18]. Mixed pattern (homogenous and speckled) has been named as Quasi homogenous as per BCA for ANA screening using HEp-2 cells and describes a pattern not included in ICAP. We have frequently observed this pattern on HEp-2 screening for ANA (13.9% in this study) lately especially post COVID-19 pandemic. The nomenclature used in our study to describe this pattern is mixed (homogenous and speckled) and is characterised by staining characteristics resembling both homogenous and speckled patterns on HEp-2 cells. The primate liver substrate offered by Euroimmun which was used in our study also showed a positive reaction which could be used to differentiate this pattern from DFS 70 which showed a similar reaction on HEp-2 cell line but does not elicit any reaction on primate liver. Table 3 depicts the distribution pattern of the four different nuclear patterns in our study.

Table 3: Pattern of distribution of the four nuclear patterns in our study.

ANA screen pattern	% distribution
DFS 70	2.1%
Homogenous	14.2%
Mixed [homogenous and speckled]	13.9%
Speckled	69.8%

Amongst the four common nuclear pattern the most common pattern noted was speckled (69.8%), a finding which is very much in agreement with previous studies. In a study by Ramachandran et al in India, the most common pattern reported was speckled (52.9%) followed by homogenous (27.5%) in a study involving 204 SLE patients [20]. In alignment with our findings, a research from Saudi Arabia found speckled pattern (79.5%) as the most frequently reported followed by the homogeneous pattern (11.4%) [21]. DFS -70 pattern is a very rarely reported pattern and its frequency across world- wide studies have varied from 0.3% to 27% [22]. DFS 70 accounted for 2.1% of the total of four nuclear patterns in our study. The DFS 70 pattern is frequently observed in people without autoimmune diseases and with a positive ANA test [22]. DFS 70 is also known to have a positive association with diseases with an autoimmune basis like Raynaud's disease and idiopathic

fibrotic alveolitis [23]. However, some studies have observed that patients with DFS 70 pattern were diagnosed with rheumatic autoimmune diseases [18]. Mixed pattern (homogenous and speckled) has been named as Quasi homogenous as per BCA for ANA screening using HEp-2 cells and describes a pattern not included in ICAP. We have frequently observed this pattern on HEp-2 screening for ANA (13.9% in this study) lately especially post COVID-19 pandemic. The nomenclature used in our study to describe this pattern is mixed (homogenous and speckled) and is characterised by staining characteristics resembling both homogenous and speckled patterns on HEp-2 cells. The primate liver substrate offered by Euroimmun which was used in our study also showed a positive reaction which could be used to differentiate this pattern from DFS 70 which showed a similar reaction on HEp-2 cell line but does not elicit any reaction on primate liver. Table 3 depicts the distribution pattern of the four different nuclear patterns in our study.

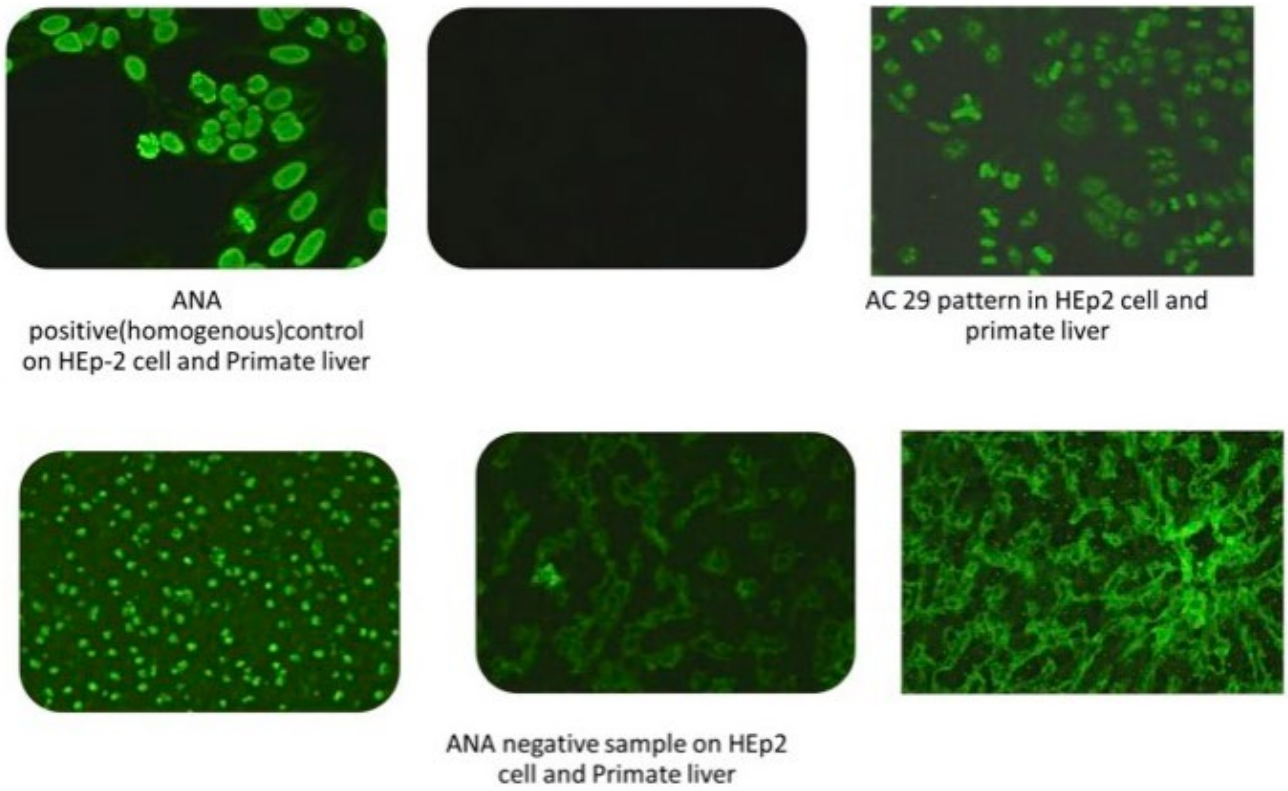
Table 4: Depicting correlation between the three nuclear patterns and antibodies detected against 16 antigens by LIA in 103 samples(DFS 70 was excluded amongst nuclear patterns as only two were positive for which LIA results were available ,CENP B and Jo-1 were excluded from ANA profile anti body list as none of the two were positive in 103 samples).

Antigens/patterns	**dsDNA**			**Nucleosome**			**Histone**		
	ABSENT	PRESENT	p value	ABSENT	PRESENT	p value	ABSENT	PRESENT	p value
homogenous	70.80%	29.20%	0.024	75%	25%	0.075	75%	25%	0.1.26
mixed	91.70%	8.30%		91.70%	8.30%		91.70%	8.30%	
speckled	92.30%	7.70%		92.30%	7.70%		90.80%	9.20%	
Antigens/patterns	**SmD1**			**PCNA**			**P0[RPP]**		
	ABSENT	PRESENT	p value	ABSENT	PRESENT	p value	ABSENT	PRESENT	p value
Homogenous	83.30%	16.70%	0.042	100%	0	0.218	91.70%	8.3%	0.599
mixed	75%	25%		91.70%	8.3%		100%	0%	
speckled	95.40%	4.60%		98.5%	1.50%		92.30%	7.7%	
Antigens/patterns	**SSA/Ro60**			**SS-A/Ro52**			**SSB/La**		
	ABSENT	PRESENT	p value	ABSENT	PRESENT	p value	ABSENT	PRESENT	p value
homogenous	87.50%	12.50%	**0.64**	91.70%	8.30%	0.362	79.20%	20.80%	0.063
mixed	75%	25%		75%	25%		66.70%	33.30%	
speckled	83.10%	16.9%		87.70%	12.30%		90.80%	9.20%	
Antigens/patterns	**Scl-70**			**U1-snRNP**			**AMA-M2**		
	ABSENT	PRESENT	p value	ABSENT	PRESENT	p value	ABSENT	PRESENT	p value
Homogenous	79.20%	20.80%	0.008	62.50%	37.5%	0.048	95.8%	4.20%	0.411
mixed	100%	0%		58.30%	41.70%		91.70%	8.3%	
speckled	96.9%	3.10%		83.10%	16.90%		98.50%	1.50%	
Antigens/patterns	**PM-Scl**			**Mi-2**			**Ku**		
	ABSENT	PRESENT	p value	ABSENT	PRESENT	p value	ABSENT	PRESENT	p value
Homogenous	95.80%	4.20%	0.458	100%	0%	0.568	75%	25%	0.48
mixed	83.30%	16.70%		100%	0%		91.70%	8.3%	
speckled	90.80%	9.2%		96.90%	3.10%		81.50%	18.50%	
Antigens/patterns	**DFS-70**								
	ABSENT	PRESENT	p value						
Homogenous	87.50%	12.50%	0.232						
mixed	91.70%	8.30%							
speckled	96.90%	3.10%							

Percentages are calculated row wise.

These findings are in agreement with other studies in past except for the homogenous pattern which was associated with Scl70 additionally in our study. Dellavance et al. in 2009 documented a composite HEp-2 pattern linked to anti-Topoisomerase I antibodies, commonly known as anti-Scl-70. The incorporation of the Topoisomerase I-like pattern into the ICAP algorithm was initially proposed at the 2nd ICAP edition at the 12th Dresden Symposium on Autoantibodies (DSA) in 2015, Dresden, Germany and at the fourth ICAP meeting in 2017, there was a consensus that the Topoisomerase I-like pattern be designated as AC-29[28]. Although autoantibodies to Topoisomerase I may be reported as homogeneous, they typically reveal a composite AC-29 HEp-2 IIF pattern and clinical suspicion of Systemic Sclerosis may call for follow-up testing for antibodies to Scl-70. In the past there have been instances of the dense fine speckled (AC-2) and topoisomerase I-like (AC-29) patterns often being considered homogeneous, speckled or even mixed patterns [4]. It is very much possible that the AC -29 pattern could have been wrongly been reported as homogenous pattern in our study due to overlapping features. AC-29, anti- topoisomerase I (previously known as Scl 70) is hence rightly placed in Expert level reporting as per ICAP ANA classification tree and calls for more awareness and expertise to be rightly reported. Figure 2 depicts positive control pattern, negative ANA sample pattern and anti-topoisomerase antibodies i.e Scl 70 (AC 29 pattern) on HEp-2 cell and primate liver obtained in our study.

Figure 2: Depicts positive ANA control, negative ANA control and anti-topoisomerase pattern (AC-29) on HEp-2 cell line and primate liver by IIF.



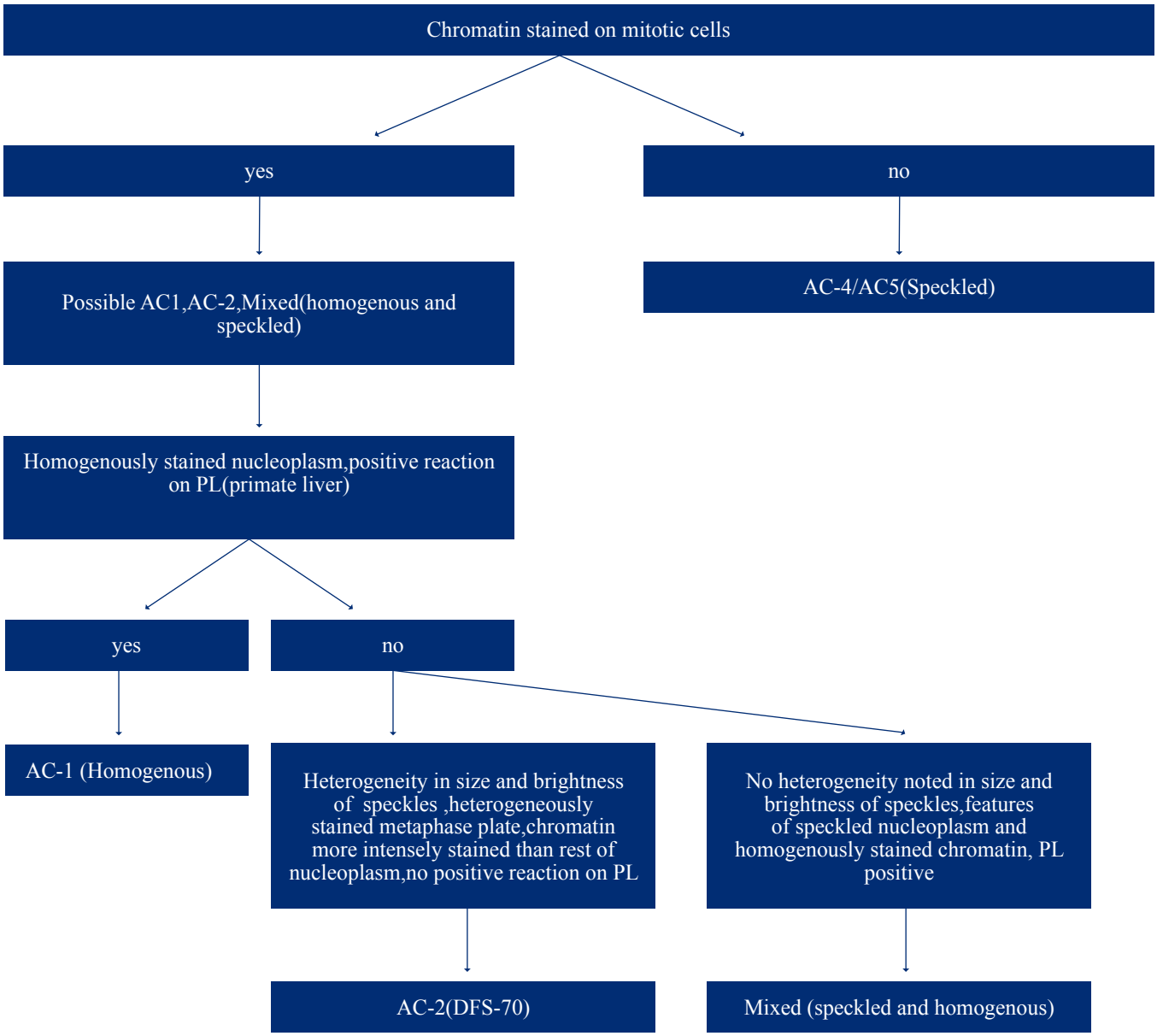
Interestingly the speckled pattern in our study, despite being the most frequently reported, failed to show association with any particular antigen detected by LIA. Mixed pattern in our study corresponded to antibodies to U1snRNP and SmD1 antigens. Antibody to U1-snRNP targets the U1snRNP.Autoreactive B cells and T cells target the U1-sn RNP in several rheumatic diseases including SLE and Mixed connective tissue disease (MCTD). In our study a total of twelve cases of mixed patterns were reported on ANA screening by HEp-2. When followed up by LIA, two of them were associated with a single antibody on LIA, six of them had more than two antibodies associated and four had no antibody associated. The mixed pattern has been associated with antibodies to U1snRNP and SmD1in our patient population and previous studies have been reported where SLE was found to be associated with two different clusters of antibodies: Sm/anti-RNP or Ro/La autoantigens. Both are proteins often involved in RNA binding activities. The Sm/RNP cluster was associated with a higher prevalence of serositis in comparison to the Ro/La cluster [29].

Elevated antibody titers against U1snRNP are also linked to MCTD. Originally defined in 1972, MCTD is characterized by the presence of anti-U1snRNP antibodies alongside overlapping clinical features reminiscent of SLE, Systemic Sclerosis, idiopathic inflammatory myositis, and rheumatoid arthritis.

U1-snRNP is one of the five snRNPs constituting the mammalian spliceosome, a crucial macromolecular complex responsible for post-transcriptional processing of pre-messenger RNA (pre-mRNA). This process involves intron removal and exon ligation to produce mature RNA for translation into proteins [30,31]. The five snRNPs—U1, U2, U4, U5, and U6—each comprise a unique small nuclear RNA molecule, specific associated proteins, and seven common core proteins known as Smith (Sm) proteins. The term "Smith protein" originates from the patient whose blood sample contained antibodies specific to the Sm complex. Autoantibodies against Sm and 'RNP', which refers to U1-specific proteins and U1-snRNA, target distinct molecular entities. While Sm proteins are present in all five snRNPs, autoantibodies against Sm precipitate all snRNP RNA molecules, whereas anti-RNP autoantibodies precipitate only U1-specific RNA, sparing other unique RNA molecules. U1-snRNP comprises U1-snRNA, the seven common core Sm proteins, and three U1-specific proteins: U1-70K, U1-A, and U1-C [30,31]. The prevalence of anti-RNP autoantibodies varies across diseases, with detection rates of 30–40% in SLE patients and nearly universal presence in MCTD patients, where high titers of anti-RNP antibodies are diagnostic criteria for MCTD [30,31]. Anti-Sm antibodies are included in the Systemic Lupus Erythematosus International Collaborating Clinics (SLICC) classification criteria for Lupus. The prevalence of anti-U1snRNP as the most common antibody on LIA, followed by Ku and the occurrence of multiple antibodies in combination suggests the likely existence of polyautoimmunity phenomena in the patient population. LIA is a platform which enables the detection of multiple autoantibodies in a single approach and may prove a valid tool in times to come.

The authors propose an algorithm based on ICAP and BCA guidelines along with their experience in approaching the identification and distinction of four nuclear patterns described above (Figure 3).

Figure 3: Algorithm suggested for evaluation of four nuclear patterns on IIF by HEP-2.



Limitations of our study

The study was carried out as a short term research for a limited duration as per guidelines of ICMR. It could have probably benefitted otherwise from a larger sample size spanning a longer duration. Moreover the number of samples included in LIA (n=103) were restricted as the work was non funded and only those samples were included for which the investigation was routinely ordered as part of patient care services. More studies in future are required to substantiate the results of this study.

Conclusions

1. Laboratory investigations play a very supportive role in the diagnosis of SARDs. Approach to laboratory investigation of a suspected case of SARD starts with IIF and is usually followed by reflex testing in positive cases using ELISA or LIA for identification of specific antigens.
2. Female predominance is noted with respect to a positive ANA screening test and the young and middle age group (20-40 years) are mostly affected.
3. Speckled pattern is the most common nuclear pattern noted in our study although no significant association with any specific antibody could be elicited in our study. Homogenous pattern was found to be most commonly associated with anti-dsDNA and anti Scl-70 antibodies while mixed pattern was associated with anti-SmD1 and antiU1snRNP antibodies.
4. Correlation of homogenous pattern with Scl-70 has not been reported very frequently in literature. Scl-70 corresponds to anti -Topoisomerase-I antibodies (AC 29). Scl-70 is a difficult pattern to identify because of its complex characteristics and is rightly placed under competent level of reporting by ICAP. This pattern deserves more attention and it may have been wrongly interpreted as homogenous (AC-1) in our study because of similarities in microscopic morphology.
5. DFS-70 which is currently placed under competent level reporting by ICAP needs to be reviewed as its identification is not devoid of challenges due to overlapping features with other three nuclear patterns namely, speckled, homogenous and mixed (homogenous and speckled). Anti-DFS 70 antibody was not reported singly in our study by LIA and was found in combination with other antibodies thus making correlation and interpretation even more difficult.
6. Mixed pattern as reported in our study mostly corresponded to antibodies against SmD1 and U1snRNP antigens. This pattern finds a mention in BCA as Quasihomogenous (features between speckled and homogenous) but is not depicted in the ANA classification tree by ICAP. This pattern has been on the rise post COVID-19 pandemic in population being catered by our hospital for SARDs and maybe addressed by ICAP.
7. Combination of multiple autoantibodies on LIA points towards the existence of polyautoimmunity in the present times highlighting the need for LIA as a detection tool.

Ethical clearance

The study has been approved by Institute Ethical committee at AIIMS Patna. The study has been completed in compliance with the ethical principles for medical research involving human subjects, in accordance with the Declaration of Helsinki.

Conflict of intertest

None declared.

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Data availability

All relevant data has been uploaded with the manuscript. Any additional data may be obtained from corresponding author on reasonable request.

Author contributions

All authors have contributed to the design and implementation of the research, to the analysis of the results and to the writing of the manuscript.

Consent for publication

All authors have consented for publication of the manuscript.

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