Original Article

DOI: 10.7860/JCDR/2023/60084.17321

Rare Antinuclear Antibody Patterns: Relevance in Routine Laboratory Reporting

Pathology Section

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ABSTRACT

Introduction: Uncommon patterns on Human Epithelial 2 (Hep 2) substrate during Antinuclear Antibody (ANA) screening by Indirect Immunofluorescence (IIF) microscopy are not routinely reported by many laboratories since their clinical relevance is not well established.

Aim: To elucidate rare ANA patterns on Hep 2 and their possible association with clinical presentation.

Materials and Methods: A retrospective study was conducted on ANA reports from January 2021 to March 2022 at the Department of Laboratory Medicine, Dr. Sulaiman Al Habib Medical Group, Olaya Medical Complex, Riyadh, Saudi Arabia, to recognise rare ANA patterns. Gold standard method of IIF on Hep 2 was used for screening. Statistical evaluation was done to obtain frequencies of various ANA patterns. Those with frequency of less than 1% were classified as rare patterns.

Results: Overall, 4207 consecutive ANA reports were evaluated out of which 1388 were positive and 210 (4.99%) demonstrated rare ANA patterns including nuclear, cytoplasmic and mitotic subtypes. Most commonly encountered among the rare ANA patterns was intercellular bridge (AC 27) with frequency of 0.78% (n=33). Systemic Lupus Erythematosus (SLE) (10/210) was the most often observed clinical association with rare cytoplasmic and mitotic patterns at titer ≥1:160.

Conclusion: Uncommon ANA patterns may be useful in initial work-up of autoimmune illness hence, should be routinely reported. Further studies to enlighten the significance of these patterns, analogous antibodies could be of diagnostic relevance in autoimmune and other diseases.

Keywords: Autoimmunity, Fluorescence microscopy, Spindle apparatus

INTRODUCTION

Antibodies against various cellular antigens, better known as ANA have been a significant screening tool in approaching patients suspected of having autoimmune illness [1]. ANA is specifically important in the evaluation and follow-up of patients with systemic autoimmune rheumatic diseases such as SLE. ANA positivity is one of the criteria set by the American College of Rheumatology (ACR) in order to classify patients with SLE [2]. Furthermore, positive ANA may not always hint to Systemic Autoimmune Rheumatic Diseases (SARD) as low titres may be found in some healthy individuals [3]. The IIF has long been considered as the gold standard screening method to detect ANA [4] which uses the Hep 2 substrate derived from human laryngeal carcinoma. Capturing monolayer cells in different phases of mitosis and allows detection of antibodies to a broad array of nuclear antigens, and those located in the cytoplasm or associated with mitotic apparatus can also be detected by this assay [5]. Based on fluorescence patterns observed, the technique enables identification of antibodies against cell antigens favourably expressed in specific mitotic phases like spindle fibre related antigen and others of unknown significance [6]. Hence, it still stays as the method of choice for initial assessment of ANA even with the emergence of commercial assays for antibodies against specific cellular antigens [7].

International Consensus on ANA Patterns (ICAP) describes 29 distinct Anti Cellular (AC) patterns named as AC 1 to AC 29 and categorised under nuclear, cytoplasmic and mitotic subtypes with defined competence level for identifying each of them. AC 0 represents as negative for any specific fluorescence for cellular autoantibodies [8-10]. Common patterns have undergone extensive research and shown to have specific disease association whereas relevance of rare patterns is still unknown due to confined studies. Rare patterns have been defined as those with prevalence of less than 1% [11]. Most patterns so far described as rare in various studies [11-14] are classified under 'expert level' category in the ICAP classification [15] and so could be the reason that some laboratories do not report them in addition to lack of certainty about their significance.

The present study was conducted in a tertiary care hospital laboratory with high turnover of patient samples for ANA screening by IIF. The study intends to analyse the range of rare ANA patterns, estimate their frequency and possible clinical relevance.

MATERIALS AND METHODS

The present retroseptive observational study was conducted at the Department of Laboratory Medicine, Dr. Sulaiman Al Habib Medical Group, Olaya Medical Complex, Riyadh, Saudi Arabia. Data for duration between 1st January 2021 to 31st March 2022 was collected retrospectively and subsequently analysed in the period 1st April 2022 to 31st July 2022. Total 4.207 ANA reports were reviewed and their respective patterns and titers were noted. Rare ANA patterns were based on frequency of less than 1% [11]. Clinical diagnoses associated with rare patterns as mentioned in medical record were noted and data was anonymously saved. No human or animal experiments were conducted in the study and all protocols were in accordance with the Helsinki Declaration of 1975.

Inclusion criteria: All ANA reports of consecutive patients referred for ANA screening by IIF in study duration were reviewed in the study.

Exclusion criteria: In case of multiple ANA requests for a patient, only the first sample was considered for study purpose and subsequent were excluded from the study.

Slide Preparation and Microscopy

ANA testing was performed using Euroimmun, Germany kits. Mosaic biochips containing two substrates Hep 2 cells and primate liver in each reaction well were incubated with diluted patient samples. Fluorescein labeled secondary antibodies bound to patient's antibodies, if present were made visible with a fluorescence microscope at 40X magnification. Serum samples were screened

at 1:80 dilution, titers were reported by serial dilutions 1:160, 1:320, 1:640. ANA reaction at 1:80 was considered as borderline and titers 1:160 and above as positive [16]. For study purpose samples showing immunofluorescence at titer 1:80 and above were considered positive and rest negative. ANA patterns were reported in accordance with the ICAP nomenclature guidelines [10]. Samples with more than one pattern also called mixed patterns were reported in the order of nuclear, cytoplasmic and then mitotic pattern [17]. Positive, negative controls were performed with each batch of testing. The laboratory is enrolled with an external proficiency program for ANA titer and pattern identification by the College of American Pathologists. The diagnoses at lower titer of 1:160 and higher titer of ≥1:320 were considered possibly relevant to rare patterns [16]. Preliminary or final diagnosis of patients reported with rare patterns were reviewed from health records and noted under two titer categories, 1:160 and ≥1:320.

STATISTICAL ANALYSIS

Descriptive statistics were used to deduce frequency (%).

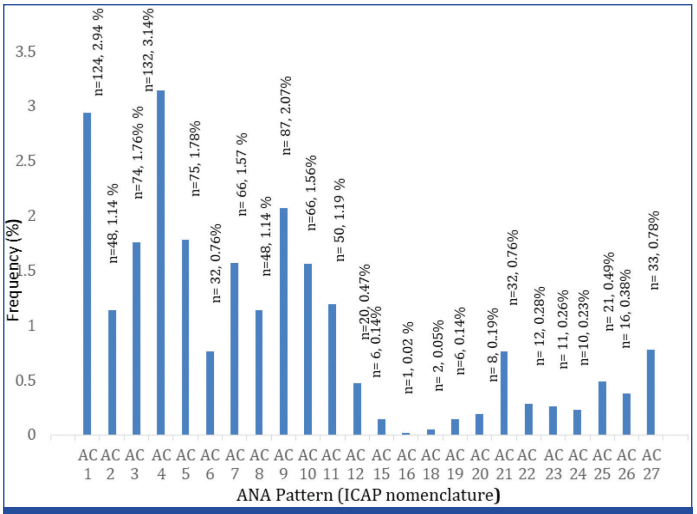
RESULTS

As depicted in [Table/Fig-1] out of 4.207 samples analysed for ANA by IIF method during the study period, 33% (n=1388) were positive. Among the positive cases, 210 (4.99%) exhibited rare ANA patterns, [Table/Fig-2] lays out the frequencies of different ANA patterns observed in the study. Rare patterns were delineated based on their observed frequencies viz., less than 1%. Overall, 24 different ANA patterns from subcategories of nuclear (n=822. 19.53%), cytoplasmic (n=78. 1.85%) and mitotic (n=80. 1.90%) were identified while mixed patterns constituted 9.70% (n=408). The most common ANA pattern was fine speckled (AC 4) with prevalence of 3.14% (n=132) whereas the rarest pattern was cytoplasmic fibrillar filamentous (AC 16) with prevalence of 0.02% (n=1).

Total 14 ANA patterns fulfilled the criterion of being rare, that is frequency of <1%. The rare patterns were from all three categories: nuclear, cytoplasmic and mitotic. The most commonly occuring rare ANA pattern was Intercellular bridge-AC 27 with frequency of 0.78% (n=33) [Table/Fig-1].

ANA pattern (ICAP nomenclature)	N (Total=4207)	Frequency (%)
Total negative (AC 0)	2819	67
Total positive	1388	33
Positive rare patterns (with frequency <1%) n=210, 4.99%		
Nuclear	Total (n=52)	
Multiple nuclear dots (AC 6)	32	0.76
Punctate nuclear envelope (AC 12)	20	0.47
Cytoplasmic	Total (n=78)	
Fibrillar linear (AC 15)	6	0.14
Fibrillar filamentous (AC 16)	1	0.02
Discrete dots (AC18)	2	0.04
Dense fine speckled (AC 19)	6	0.14
Fine speckled (AC 20)	8	0.19
Reticular/AMA (AC 21)	32	0.76
Polar/Golgi (AC 22)	12	0.28
Rods and rings (AC 23)	11	0.26
Mitotic	Total (n=80)	
Centrosome (AC 24)	10	0.23
Spindle Fiber (AC 25)	21	0.49
NuMA like (AC 26)	16	0.38
Intercellular bridge (AC 27)	33	0.78
Positive: Other common patterns (Frequency >1%) n=1178, 28%		

[Table/Fig-1]: Frequency of rare ANA patterns in the study.
*ICAP: International consensus on ANA patterns; AC: Anticellular; N: Number



[Table/Fig-2]: Frequency of all ANA patterns observed in study.

As reflected in the [Table/Fig-3] the most frequently encountered autoimmune disorder in patients with rare ANA patterns was SLE, Other associations were of Rheumatoid Arthritis (RA), Autoimmune Hepatitis (AIH), Primary Biliary Cholangitis (PBC) and Mixed Connective Tissue Disease (MCTD). Higher titers of ≥1:320 were more frequently related to these diagnoses.

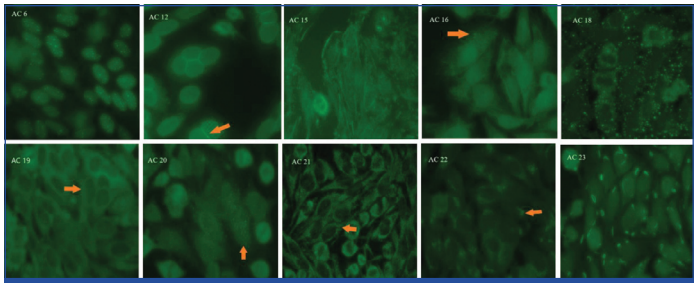
IIFT pattern	Titre	Prediagnosis/ Diagnosis	N=175	N (Titre 1:80)=35	Total N=210
Multiple nuclear dots (AC 6)	1:160	Infections (10), Non specific dermatitis (4), Hypothyroidism (3), Neoplasia (1)	18	10	32
	≥1:320	Asthma (1), PBC (1), Infection (2)	04		
Punctate nuclear envelope (AC 12)	1:160	Pneumonia (2), Viral hepatitis (3), AIH (1)	06	07	20
	≥1:320	PBC (1), Chronic lung disease (1), Infection (3), Fatty liver (2)	07		
Fibrillar linear (AC 15)	1:160	Hepatitis (1), Pneumonia (1), Urinary tract infection (1)	3	0	6
	≥1:320	Cirrhosis (1), Neoplasm (1), AIH (1)	3		
Fibrillar Filamentous (AC 16)	1:160	Non specific skin rash (1)	1	0	1
	≥1:320	-	0		
Discrete dots (AC18)	1:160	-	0	0	2
	≥1:320	Chronic back pain (1), Spondylolisthesis (1)	2		
Dense fine speckled (AC 19)	1:160	Infections (3), SLE (1)	4	1	6
	≥1:320	SLE (1)	1		
Fine speckled (AC 20)	1:160	Infections (3), Arthralgia (1), ILD (1), Non specific skin (2)	7	0	8
	≥1:320	Myositis (1)	1		
Reticular/ AMA (AC 21)	1:160	Fatty liver (8), Infections (2), Neoplasm (1), PBC (1)	12	2	32
	≥1:320	Hepatitis (4), PBC (1), Infection (5), Skin rash (3), Hypertension (1), other (4)	18		

Golgi (AC 22)	1:160	Skin Rash (1), DVT (3), Hepatitis (4),	8	0	12
	≥1:320	Bullous pemphigoid (1), RA (1), Infection (2)	4		
Rods and rings (AC 23)	1:160	Hypertension (1), RA (1), Non specific skin rash (2), Infection (1), Neoplasm (1)	6	0	11
	≥1:320	Infections (2), Skin rash (1), RA (1), Fatty liver (1)	5		
Centrosome (AC 24)	1:160	Infections (3), Other (2)	5	3	10
	≥1:320	Allergic dermatitis (1), Other liver disease (1)	2		
Spindle fibers (AC 25)	1:160	Infections (4), Skin rash (3), Nephropathy (1), Musculoskeletal (1)	9	5	21
	≥1:320	SLE (2), Other musculoskeletal disorder (1), Infection (2), Other (2)	7		
NuMA like (AC 26)	1:160	Arthritis (2), Myalgia (1), Goiter (1),SLE (1), Other (3),	8	1	16
	≥1:320	Miscarriage (2), SLE (2), Skin rash (2), Arthritis (1)	7		
Intercellular bridge (AC 27)	1:160	Neoplasm (1), Infections (4), Polyneuropathy (1), Miscarriage (1), Goiter (1), Other (3)	11	6	33
	≥1:320	SLE (3), Vasculitis (2), RA (2), Neoplasm (1), Infections (4), Skin rash (2), Alcoholic liver disease (1), MCTD (1)	16		

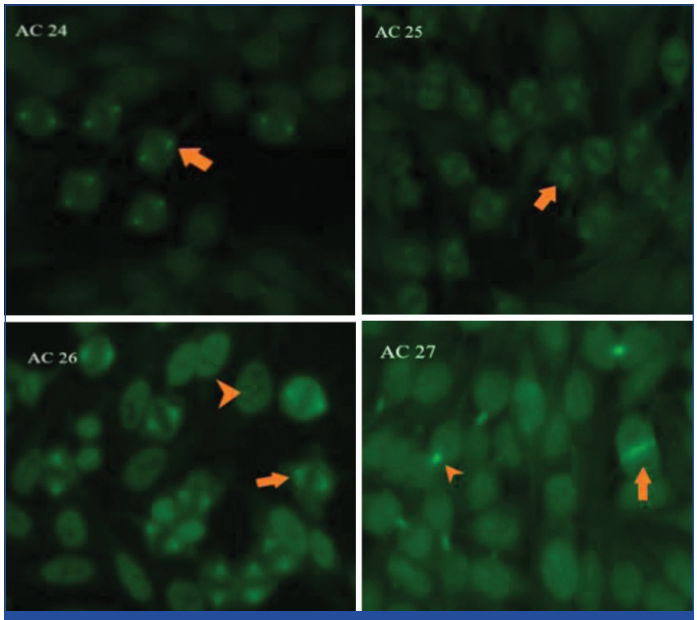
[Table/Fig-3]: Rare AC patterns with corresponding prediagnosis/diagnosis.
*n, N: Number; PBC: Primary biliary cholangitis; AIH: Autoimmune hepatitis; SLE: Systemic lupus erythematosus; ILD: Interstitial lung disease; DVT: Deep venous thrombosis; RA: Rheumatoid arthritis; MCTD: Mixed connective tissue disease

The SLE could be linked to cytoplasmic and mitotic patterns and so did RA that exhibited cytoplasmic (AC 22, 23) and mitotic patterns (AC 27). Autoimmune liver disease namely, PBC and AIH were possible diagnoses related to patterns AC 6, 12, 15, 21 while MCTD was linked to AC 27.

[Table/Fig-4] shows immunofluorescence microscopic images of rare nuclear- AC 6, AC 12 [Table/Fig-4a,b] and cytoplasmic ANA patterns- AC 15, 16, 18, 19, 20, 21, 22, 23 [Table/Fig-4c-j] on Hep 2 substrate, as observed in the study. [Table/Fig-5] shows the images of rare mitotic ANA patterns-AC 24, 25, 26, 27 [Table/Fig-5a-d], as noted in the study



[Table/Fig-4]: Fluorescence microscopy images for ANA rare patterns (40X, Hep 2 substrate).
Top Left to Right: (4a) AC 6, Multiple nuclear dots; (4b) AC 12, Punctated nuclear membrane, arrow shows enhanced fluorescence when two membranes touch; (4c) AC 15, Cytoplasmic Fibrillar Linear; (4d) AC 16, Cytoplasmic Fibrillar Filamentous, arrow showing fine net of fibers in cytoplasm; (4e) AC 18, Cytoplasmic Discrete dots.
Bottom Left to Right; (4f) AC 19, Dense fine speckled; (4g) AC 20, Fine Speckled; (4h) AC 21, Fibrillar Linear; (4i) AC 22, Golgi, arrow showing granular staining adjacent to one side of nucleus; (4j) AC 23, Rods and rings



[Table/Fig-5]: Fluorescence microscopy images of mitotic rare patterns (40X, Hep 2 substrate):
Top Left to Right: (5a) AC 24- Centrosome; (5b) AC 25- Spindle fibers
Bottom Left to right; (5c) AC 26- NuMa Like, arrowhead shows fine speckled staining of interphase nuclei which is absent in AC 25; (5d) AC 27 shows a fluorescing dot (arrowhead) between daughter cells in telophase called "Goodbye kiss"

DISCUSSION

The retrospective analysis of ANA reports in the study duration showed 4.99% rare patterns which was comparable to another similar study that reported it to be 6.39% [14]. Present study included rare patterns among all categories- nuclear, cytoplasmic, mitotic and demonstrated possible association with autoimmune diseases both at low and high titers.

Nuclear pattern: Multiple Nuclear dots (AC 6) and punctate nuclear envelope (AC 12) were the rare types among nuclear patterns. AC 6 pattern has been mainly associated with PBC however, out of total cases reported with this pattern, only one was diagnosed with PBC. The pattern was also noted in some patients with pneumonia, upper respiratory tract infection and conjunctivitis. Infectious agents are considered possible triggers of autoimmunity [18] and still remain a topic of interest to various scientists. Nuclear envelope pattern is classified further as Smooth (AC 11) and punctate (AC 12). In this study, 20 patients had AC 12 pattern out of which 2 patients were diagnosed with autoimmune liver disease. A follow-up testing for antiglycoprotein-210 antibodies is however recommended, other target antigens for this pattern include p62 nucleoporin, lamin B receptor [19].

Cytoplasmic pattern: All the cytoplasmic patterns observed in the study fulfilled the criterion of being a rare pattern and most frequent among all was Reticular/AMA like (AC 21). AC-21 has been associated with PBC and antibodies are directed against the E2 subunit of PDH complex [19]. In present study, two cases with this pattern had PBC while eight other cases were suspected of liver disease. Fibrillar Linear (AC 15) is commonly found in patients with AIH 1 and F actin being the main target antigen of Anti Smooth Muscle Antibodies (ASMA) associated with this pattern. Only one case had an established diagnosis of AIH. Both fibrillar filamentous (AC 16) and Discrete dots (AC 18) are not typically associated with autoimmune disorders and present study too did not reveal any relationship. Similar to this study Nanda R et al., also did not report association of AC 16 with SARD [14].

There is a fine distinction between dense fine speckled (AC 19) and fine speckled (AC 20) that may depend on detection of anti Jo1 antibodies primarily linked to AC 20, although not specific [19]. The AC 19 may be found in SLE as in present study, two cases showed this pattern. The antigens recognised are Ribosomal P phosphoproteins, included under Extractable Nuclear Antigen (ENA) profile, which were not done in the observed two cases however, dsDNA antibodies were positive. Tomić Sremec N et al., reported several antibodies associated with AC 19 pattern including antids DNA, antiTRIM21, antihistones and antiribosomes and all may be present in SLE [11]. AC 20 may be found in patients with antisynthetase syndrome, Interstitial Lung Disease (ILD), polyarthritis [19]. In present study, one patient with myositis showed AC 20, antiJo1 antibodies associated with this pattern, directed to histidyl t RNA synthetase, were also positive. Another patient exhibiting AC 20 was diagnosed as ILD however autoimmune aetiology was not further confirmed. Patients with RA and Bullous Pemphigoid showed fluorescence for Polar/Golgi Like pattern (AC 22) at high titre. First described in a patient with lymphoma and Sjogren syndrome, the pattern cannot be indicative of a specific autoimmune disease in current clinical practice [20] however, one study mentioned that the pattern may be an early sign of future autoimmune disease [14]. Rods and rings pattern (AC 23) has been described in patients on ribavirin/interferon treatment for Hepatitis C virus infection, the target antigen being inosine-5'-mono- phosphate dehydrogenase 2 [19,21]. Though, none of the patients in this study had Hepatitis C infection two were suspected of RA, similar to a recent case series report where the pattern has been described in various other diseases including autoimmune [22].

Mitotic pattern: These patterns mark the antigens associated with mitotic apparatus. The antibodies directed against various antigenic markers of mitotic apparatus have not been identified well but the clinical association of corresponding ANA patterns reported in previous literature makes them crucial markers of SARD [23]. The intercellular bridge or Midbody pattern (AC 27) was found to be the commonest among all rare patterns in current study. It is the residual part of dividing daughter cells just before separation and has been associated with systemic Sclerosis and malignancies [14,21]. Nanda R et al., have reported a strong association with autoimmune aetiology [14], similar to the present study where patients with SLE, RA and MCTD exhibited this pattern. Spindle Fiber (AC 25) was the next commonly encountered mitotic pattern. Two of the SLE patients exhibited this pattern which corroborates with another study where the pattern has demonstrated association with SLE [14].

NuMA-like pattern (AC 26) is found in SARD. NuMA represents several proteins involved in the nuclear reconstruction after mitosis and spindle microtubule organisation [24]. Out of total, three patients with AC 26 manifested as SLE. In a recent study, NuMA was found to be significant in patients with cardiovascular disease and autoantibodies probably pointed to coexisting autoimmune disease or an underlying autoimmune process leading to heart disease [24]. Centrosome pattern (AC 24) is characterised by fluorescence of 1 or 2 centrioles at the opposing poles and is mostly described in non specific infections. The present study did not find any possible association with SARD although Vermeersch P et al., have reported AC 24 in Raynaud's [21] and another study found it in patients with small vessel vasculitis [14].

Limitation(s)

The major limitation was the retrospective nature which was the reason for incomplete diagnoses in some cases and hence some rare ANA patterns could not be related to any disease. Also, there were slight variations in ANA pattern-disease associations compared to some previously published studies which could be attributed to ethnic diversity. A single centre study results in lack of representation of a varied population and hence could have led to a bias.

CONCLUSION(S)

With increasing worldwide prevalence of autoimmune diseases, it seems pertinent to implement effective screening for early diagnosis as timely intervention could bring better outcomes in these patients and ANA by immunofluorescence, being the gold standard, could be an effective tool. Rare ANA patterns, especially cytoplasmic and mitotic patterns, which are not routinely reported, could also be of significance in diagnosis of autoimmune diseases as evinced in present study and hence should be identified and reported in routine laboratory practice. Though, it is essential to investigate further through prospective studies to support the existing finding.

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PLAGIARISM CHECKING METHODS: [Jain H et al.]
- Plagiarism X-checker: Sep 19 2022
- Manual Googling: Nov 17, 2022
- iThenticate Software: Nov 24, 2022 (4%)

ETYMOLOGY: Author Origin

AUTHOR DECLARATION:
- Financial or Other Competing Interests: None
- Was Ethics Committee Approval obtained for this study? No
- Was informed consent obtained from the subjects involved in the study? NA
- For any images presented appropriate consent has been obtained from the subjects. NA

Date of Submission: **Sep 17, 2022**
Date of Peer Review: **Nov 10, 2022**
Date of Acceptance: **Nov 25, 2022**
Date of Publishing: **Jan 01, 2023**