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Microbiology Section

Analysis of Antinuclear Antibody Test Referral Patterns in a Tertiary Care Hospital over Three Years: A Retrospective Observational Study

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ABSTRACT

Introduction: Antinuclear Antibodies (ANA) detection by Human Epithelial Substrate (Hep2) is the recommended screening test for the diagnosis of ANA-Associated Rheumatic Diseases (AARD). ANA is ordered by various specialists in a tertiary care hospital, and a positive ANA result is followed by testing for specific autoantibodies. High pretest probability, pattern type with intensity, and referral departments are key factors determining the autoimmune diagnosis.

Aim: To analyse the ANA referral patterns among the various departments and also to estimate the prevalence and clinical significance of rare ANA patterns.

Materials and Methods: A retrospective observational study was conducted at Nizam's Institute of Medical Sciences, Hyderabad, a tertiary care hospital, in Hyderabad, Telangana, India where ANA test reports (n=16,994) from various departments over three years (November 2017 to October 2020) were evaluated between November 2022 to December 2022. ANA tests were performed on Hep2 substrate at a 1:100 dilution, and ANA patterns were reported according to the International Consensus on ANA Patterns (ICAP) nomenclature. Statistical analysis of department-wise ANA positivity and fluorescence intensity was conducted, and the final diagnoses of patients with rare ANA patterns (<1%) were noted from clinical records. Fischer's-exact test was used for comparing categorical variables, considering p-value <0.05 as statistically significant.

Results: The majority of ANA requests were from the Rheumatology department (5859; 34.5%), followed by nephrology (3132; 18.4%), neurology (1940; 11.4%), general medicine (1646; 9.7%), haematology (1106; 6.5%), and casualty (878; 5.2%), accounting for 85.6% of total referrals. The highest percentage of positivity among ANA referrals was observed in Rheumatology (333; 5.7%), with 58% of positive ANA showing 4+ intensity. No positives were observed from many surgical departments. Rare ANA patterns with a prevalence of less than 1% were observed in 22 patients with mitotic patterns accounting for the majority of rare patterns seen in 11 out of 22 (50%) cases followed by nuclear envelope, rods rings, and nuclear dense fine speckled patterns were observed in six, four, and one patient(s), respectively. The majority of rare ANA patterns had 2+ fluorescence intensity without any associated autoimmune diagnosis.

Conclusion: The highest and lowest positivity among ANA referrals were observed in the rheumatology and surgical departments, respectively. Considering the pretest probability of AARD before ordering an ANA test would lead to the optimum utilisation of laboratory services. Mitotic patterns constituted the majority of rare ANA patterns and need to be clinically correlated with antibody titers.

Keywords: Indirect immunofluorescence, Mitotic patterns, Pretest probability, Rare antinuclear antibodies patterns

INTRODUCTION

Antinuclear Antibody (ANA) testing using indirect Immunofluorescence (IIF) is the recommended screening test for diagnosing autoimmune diseases [1,2]. ANA patterns are reported according to the International Consensus on ANA patterns (ICAP) and are designated as AC 1 (anti-cell) to AC 29 [3,4]. The clinical significance of common patterns such as speckled, homogeneous, and nucleolar is well-documented and strongly correlates with established clinical criteria for corresponding autoimmune diseases. ANA tests are requested by various departments in tertiary care settings, and a positive ANA is followed by individual ELISA or line immunoassay tests for various autoantibodies based on clinical criteria and reported pattern. However, confirmatory tests are not necessary for certain ANA patterns, including a few nuclear dots, low-titre nucleolar, spindle fibers, Nuclear Mitotic Apparatus (NuMA), intercellular bridge, CENP-F-like, cytoplasmic GW bodies, polar/Golgi-like, and cytoplasmic filamentous/microtubules [5].

Some patterns are designated as "expert level" due to their infrequent appearance, uncertain clinical relevance, and difficulty in identification [4,6]. Expert level ANA patterns are rare, but some of them have clinical significance at higher antibody titers. Recent studies have reported that rare ANA patterns (<1% prevalence) occur at frequencies of 4.99% and 6.39% [7,8]. Since ANA is a screening test, it can lead to false positives if the clinical criteria associated with the disease are not met before ordering the test [9-11]. Additionally, ANA has been found to be present in 20 to 30% of the general population [12], and there is evidence of increasing ANA positivity with advancing age [13-15]. These findings suggest that ANA test ordering should be restricted to cases with a high pretest probability of Systemic Autoimmune Rheumatic Disease (SARD).

The present study aimed to retrospectively analyse the ANA referral patterns in a tertiary care hospital, focusing on department-wise percentage positivity, pattern types, and fluorescence intensity. Another objective was to estimate the number of rare ANA patterns and examine the association between autoimmune clinical diagnoses and fluorescence intensity in those patients.

MATERIALS AND METHODS

The present retrospective observational study was conducted at the Department of Microbiology, Nizam's Institute of Medical Sciences in Hyderabad, Southern India. Data from the period between November 2017 and October 2020 were retrospectively collected and analysed between November 2022 to December 2022. A total of 16,994 ANA results were reviewed and data on department-wise ANA positivity, fluorescence intensity, and pattern were recorded. ANA patterns with a prevalence of less than 1% were categorised as

"rare patterns" [16,17], and available clinical records were reviewed to identify possible autoimmune diagnoses.

Since this was a retrospective study analysing only clinical details without personal information or any additional procedures or sample collection from patients, Institutional Ethics Committee (IEC) approval was not obtained.

Inclusion criteria: All consecutive patients referred for ANA testing during the study period were included, and their ANA results were reviewed.

Exclusion criteria: For patients with multiple ANA test requests, only the first result was considered, and subsequent results were excluded from the study.

Sample size calculation: The ANA prevalence in the Indian population was considered as 33% based on a previous study by Gupta P et al., and Charan J et al., [18,19]. The sample size was calculated using the formula $Z^2 \times p(1-p)/d^2$, where the Z-value was 1.96 for a 95% confidence interval, the prevalence (p) was 0.33, and the precision (d) was 0.05 [19]. The minimum sample size required was found to be 340 patients, compared to the 16,994 results analysed. Since this was a retrospective analysis of routine ANA referrals, the disadvantages of oversampling, such as unnecessary intervention and resource wastage, did not apply to the present study.

Study Procedure

Serum samples were tested using indirect IIF with Euroimmun Mosaic HEp-20-10 and primate liver cell substrate (Euroimmun AG, Germany, Lübeck) at a 1:100 dilution. All slides were observed under a fluorescent microscope by two independent observers, and the patterns were reported according to ICAP nomenclature [18,20]. Fluorescence intensity was graded from 1+ to 4+, comparing it with the intensity of the positive control (4+). The maximum intensity with brilliant green fluorescence was graded as 4+, less brilliant green fluorescence as 3+, definite but dull green fluorescence as 2+, and very dim subdued fluorescence as 1+ [21]. ANA patterns with a prevalence of less than 1% were considered rare patterns, and the clinical records of these patients were evaluated for possible autoimmune diagnoses.

STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad Prism statistical software, Version 9.5.0 (730). Categorical variables such as department-wise ANA positivity and fluorescence intensity were compared using Fisher's test. A p-value <0.05 were considered significant.

RESULTS

A total of 16,994 samples were tested for ANA during the study period. ANA was positive in 728 patients (4.28%), with nuclear patterns being the most common in 546 (75%) [Table/Fig-1].

ANA pattern	Number (%)
Nuclear	546 (75%)
Cytoplasmic	159 (21.9%)
Mitotic	23 (3.1%)

[Table/Fig-1]: Distribution of positive ANA patterns.

The majority of the referrals were from the Rheumatology department- 5,859 (34.5%), followed by Nephrology- 3,132 (18.4%), Neurology- 1,940 (11.4%), General Medicine- 1,646 (9.7%), Haematology- 1,106 (6.5%), and Casualty- 878 (5.2%), accounting for 85.6% of the total referrals. Departments with a minimum of 100 ANA requests were analysed for ANA positivity and pattern types, as shown in [Table/Fig-2].

None of the ANA requests from surgical departments such as neurosurgery, surgical gastroenterology, urology, and plastic surgery were positive.

Most of the ANA referrals were from patients below the age of 65 years (16,196; 95.3%), and ANA positivity was slightly higher in patients >65 years (5.3% vs. 4.2%) [Table/Fig-3].

Among the rare nuclear ANA patterns, Dense fine speckled (AC-2) and Smooth nuclear envelope (AC-11) were noted in one and six patients respectively. Rods and rings pattern (AC-23) was the only rare cytoplasmic pattern observed in four patients [Table/Fig-4].

ANA patterns AC24 to AC28 are designated as Mitotic patterns according to ICAP nomenclature, and they are caused by autoantibodies targeting cell cycle-related antigens. The Centrosome pattern (AC24) is characterised by the presence of two distinct centrioles at the poles of the mitotic spindle, which is caused by pericentrin and ninein antigens. In the Spindle fibers pattern (AC25), the spindle fibers between the poles are stained in mitotic cells, accompanied by a cone-shaped decoration of the mitotic poles, which is caused by the HsEg5 antigen. The NuMA-like pattern (AC26) shows speckled nuclear staining along with staining of spindle fibers, attributed to the NuMA antigen. The Intercellular bridge pattern (AC27) is characterised by staining of the intercellular bridge before the separation of daughter cells, with no particular antigen association. The mitotic chromosomal pattern (AC28) displays staining of the metaphase plate without staining of interphase cells, and it is caused by the modified histone 3 antigen, as depicted in [Table/Fig-5].

Referring department	Total ANA requests (total N=16994) n (%)	Total ANA positives (total N=728) n (%)	Distribution of ANA positives			Negative (total N=16266) n (%)
			Nuclear pattern (total N=546) n (%)	Mitotic pattern (total N=23) n (%)	Cytoplasmic pattern (total N=159) n (%)	
Rheumatology	5859 (34.5)	333 (5.7)	262 (78.7)	6 (1.8)	65 (19.5)	5526 (94.3)
External referrals	706 (4.2)	39 (5.5)	30 (76.9)	4 (10.3)	5 (12.8)	667 (94.5)
Casualty	878 (5.2)	44 (5)	33 (75)	0	11 (25)	834 (95)
Gastroenterology	442 (2.6)	19 (4.3)	12 (63.2)	0	7 (36.8)	423 (95.7)
Pulmonary medicine	457(2.7)	19 (4.2)	12 (63.2)	0	7 (36.8)	438 (95.8)
Haematology	1106 (6.5)	45 (4.1)	34 (75.6)	1 (2.2)	10 (22.2)	1061 (95.9)
Cardiology	158 (0.9)	6 (3.8)	4 (66.7)	0	2 (33.3)	152 (96.2)
General medicine	1646 (9.7)	57 (3.5)	38 (66.7)	4 (7)	15 (26.3)	1589 (96.5)
Nephrology	3132 (18.4)	107 (3.4)	79 (73.8)	4 (3.7)	24 (22.4)	3025 (96.6)
Medical genetics	164 (0.9)	5 (3)	5 (100)	0	0	159 (97)
Neurology	1940 (11.4)	44 (2.3)	33 (75)	4 (9)	7 (15.9)	1896 (97.7)
Dermatology	137 (0.8)	0	0	0	0	137 (100)
Surgical departments	245 (1.4)	3 (1.2)	1 (33.3)	0	2 (66.7)	242 (98.7)
Other medical departments	124 (0.7)	7 (5.6)	3 (42.9)	0	4 (57.1)	117 (94.3)

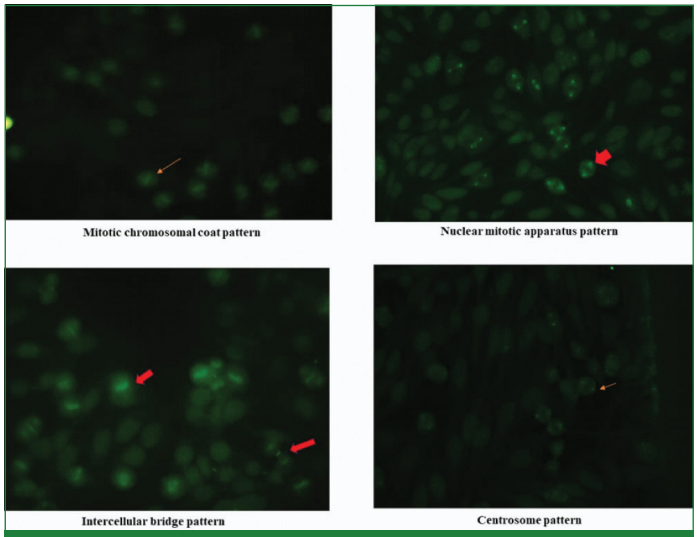
[Table/Fig-2]: Department wise ANA referrals with ANA positivity.

Age group-years	Total ANA N=16994 (%)	ANA positive N=728 (%)	ANA negative N=16266 (%)
<34	7974 (47%)	368 (4.6%)	7606 (95.4%)
35-44	3766 (22.1%)	157 (4.2%)	3609 (95.8%)
45-54	2960 (17.4%)	116 (3.9%)	2844 (96.1%)
55-64	1496 (8.8%)	45 (3%)	1451 (97%)
65-74	666 (3.9%)	37 (5.6%)	629 (94.4%)
75-84	125 (0.7%)	5 (4%)	120 (96%)
85-94	7 (0.04%)	0 (0%)	7 (100%)

[Table/Fig-3]: Age wise distribution of ANA referrals.

ANA pattern	Number (total ANA positives, n=728)	Percentage (%) of prevalence
Mitotic chromosomal pattern (AC-28)	1	0.1
Dense fine speckled (AC-2)	1	0.1
Nuclear mitotic apparatus (AC-26)	2	0.3
Intercellular bridge (AC-27)	4	0.5
Rods and rings (AC-23)	4	0.5
Centrosome (AC-24)	4	0.5
Smooth nuclear envelope (AC-11)	6	0.8

[Table/Fig-4]: Distribution of rare ANA patterns.



[Table/Fig-5]: Fluorescence images of rare mitotic ANA patterns. Top left to right: Mitotic chromosomal pattern shows staining of the metaphase plate with no staining of interphase cells. NuMA pattern displays staining of the spindle poles. Bottom left to right: Intercellular bridge pattern exhibits staining of the intercellular bridge between daughter cells. Centrosome pattern reveals staining of two centrioles at the poles of the mitotic spindle.

The non mitotic rare patterns included two nuclear patterns: Dense fine speckled (AC-2) and smooth nuclear envelope (AC-11), as well as a cytoplasmic pattern called rods and rings (AC-23).

Out of the total rare patterns (22), half of them (11/22) were mitotic patterns, while the remaining non mitotic patterns were predominantly observed in the rest of the patients. This difference was statistically significant [Table/Fig-6].

Rare vs other patterns	Type of ANA pattern		Total	p-value*
	Mitotic ANA patterns	Non mitotic ANA patterns		
Rare patterns	11	11	22	<0.0001 (statistically significant)
Other patterns	0	706	706	
Total	11	717	728	

[Table/Fig-6]: Distribution of rare patterns among mitotic and non-mitotic ANA patterns.
*Fischer's-exact test; p-value <0.05 was considered significant

The analysis of ANA positive data from departments with more than 100 ANA requests (n=718) revealed that over 50% of ANA positive samples from rheumatology, pulmonary medicine, and External Referrals displayed a 4+ intensity, as shown in [Table/Fig-7].

Department	4+ Intensity (%)
External (23/39)	59.0
Rheumatology (193/333)	58.0
Pulmonary medicine (11/19)	57.9
General medicine (28/57)	49.1
Casualty (21/44)	47.7
Nephrology (49/107)	45.8
Gastroenterology (8/19)	42.1
Medical genetics (2/5)	40.0
Haematology (16/45)	35.6
Cardiology (2/6)	33.3
Neurology (12/44)	27.3

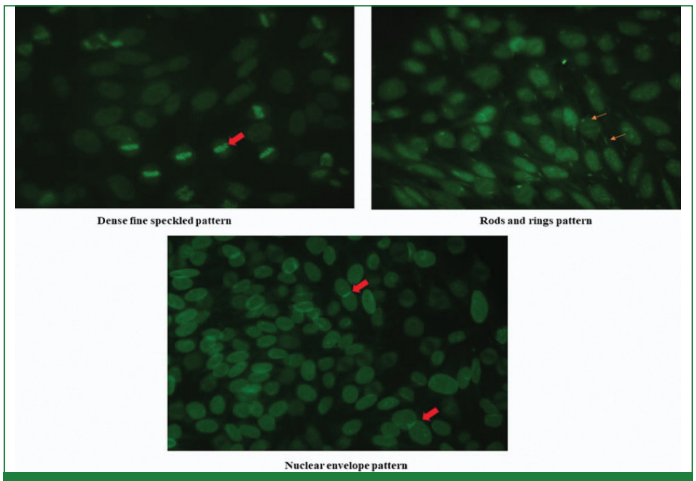
[Table/Fig-7]: Department wise comparison of fluorescence intensity among positive ANA results.

A significant proportion of ANA positive samples displaying a 4+ intensity were from the Rheumatology department (52.8%, 193/365) compared to other departments (172/365 47.12%) (p-value=0.0004), as shown in [Table/Fig-8].

Department	Fluorescence intensity		Total	p-value*
	4+ fluorescence intensity	<4+ fluorescence intensity		
Rheumatology	193	140	333	0.0004 (statistically significant)
Other departments	172	213	385	
Total	365	353	718	

[Table/Fig-8]: Comparison of fluorescence intensity among departments with more than 100 ANA referrals (n=718).
*Fischer's-exact test; p-value <0.05 was considered significant

All six patients with the nuclear envelope pattern (AC-11) had an autoimmune liver disease and showed a 4+ fluorescence intensity. The nuclear envelope pattern is characterised by accentuated fluorescence at the junction of two adjacent cells and is caused by antibodies against lamin-associated proteins. The remaining rare non mitotic patterns had a fluorescence intensity of 2+, except for one patient with a dense fine speckled pattern who had a 3+ intensity. These patterns were not associated with any autoimmune disease, as depicted in [Table/Fig-9].



[Table/Fig-9]: Fluorescence images of rare non mitotic ANA patterns. Top left to right: The dense fine speckled pattern shows a brightly stained speckled metaphase plate with dull nucleoplasm. In the rods and rings pattern, there are rod and ring structures present in the cytoplasm of interphase cells. Bottom: The nuclear envelope pattern is characterised by an accentuation of fluorescence at the junction of two adjacent cells.

DISCUSSION

ANA tests are ordered by various specialists aside from Rheumatologists. However, a positive ANA test result needs to be

correlated with the patient's clinical condition and specific disease criteria for autoimmune diseases. In the present study, the majority of ANA tests and positive results were from the Rheumatology department. A study conducted in the United States of America (USA) over a period of two years found that over 90% of patients referred to a tertiary rheumatology clinic for a positive ANA test result showed no evidence of an AARD [9].

Numerous studies have shown a high prevalence of ANA positivity in both the general population and various patient populations [22-26]. ANA positivity has been reported to increase with age [15]. In the present study, although the majority of referrals were from patients under 65-year-old, a slightly higher positivity rate was observed in the elderly population (5.3%). ANA frequencies among the elderly range from 10-37% [13], and the specificity and positive predictive value of ANA testing in elderly patients have been reported to be lower compared to younger patients [14].

In the present study, rare ANA patterns with a prevalence of less than 1% were predominantly mitotic patterns. A recent multicentric Spanish study concluded that mitotic patterns did not show any preference for a specific disease, with 62.7% of them corresponding to the NuMA1 pattern (AC-26) [26]. Several studies conducted worldwide have reported varying prevalence of mitotic and other rare ANA patterns [Table/Fig-10] [8,16,27-29].

Author/Study parameters	Betancur JF et al., 2018 [27]	Vermeersch P and Bossuyt X, 2013 [16]	Szalat R et al., 2010 [28]	Nanda R et al., 2021 [8]	Sener AG 2018 [29]	Present study, 2023
Place of study	Columbia	Belgium	France	Raipur, Central India	Turkey	Southern India
Total ANA tested	113491	68128	36498	1235	41921	16994
Total positive N (%)	60501 (53)	9268 (13.6)	10585 (29)	330 (26.7)	9908 (23.6)	728 (4.28)
Nuclear Mitotic Apparatus (NuMA)	0.46%	0.71	0.26	0.6	-	0.3
Intercellular bridge	0.32%	0.32	-	1.8	0.49	0.5
Centrosome	0.17%	0.1	-	0.6	0.29	0.5
Spindle fibres	-	0.06	0.12	3.3	0.2	1.68
MND*	0.13%	-	-	2.7	-	-
CENP-F†	0.01%	-	-	-	-	-
Nuclear envelope	0	0.52	-	0.6	-	0.8
Golgi	0.03	0.37	-	1.8	0.21	3.02
PCNA‡	0.03	0.3	-	-	0.09	-

[Table/Fig-10]: Comparison of prevalence of rare ANA patterns from various studies [8,16,27-29].
*Multiple nuclear dots; †Centromere protein F; ‡Proliferating cell nuclear antigen

The Centrosome (AC24) pattern is a very rare pattern associated with autoimmune diseases at high titers [22]. It has been observed in patients with different systemic autoimmune disorders including Sjogren's syndrome, Systemic lupus erythematosus, Scleroderma, Raynaud's phenomenon, as well as in patients with viral or mycoplasmal infections [30]. The Intercellular bridge pattern (AC27) has no specific antigen association and has been reported to be associated with cancer at higher dilutions (≥640) [9]. NuMA1 (AC26), although reported to be associated with systemic autoimmune diseases in various studies [28,31], was not associated with autoimmune disease in the present study, possibly due to low titers as both samples displayed a 2+ intensity.

The mitotic chromosomal pattern (AC28) was the rarest pattern in the present study, observed in only 0.1% of ANA positive patients. It has been found to have a low predictive value for any specific disease. The majority of the rare ANA patterns in the present study were mitotic patterns with a 2+ fluorescence intensity and had no associated autoimmune diagnosis. Various studies have suggested that the likelihood of AARD increases with increasing fluorescence intensity [32-34].

The dense fine speckled pattern is rarely seen in AARD and is more commonly observed in healthy individuals [35-37]. Anti-DFS70 antibodies in apparently healthy individuals have been reported to range from 0-21.6% [38]. The Rods and rings pattern is a cytoplasmic pattern that is more commonly reported in Hepatitis C Virus (HCV) positive patients treated with Ribavirin or Interferon [39-41]. However, it can also be observed in HCV negative patients [42,43].

The nuclear envelope pattern was the only rare pattern with a 4+ intensity that was associated with autoimmune liver disease in the present study, as observed in another study as well [16]. Some studies have suggested that higher antibody levels are better associated with AARD and have an increased likelihood of identifying autoantigens in follow-up testing [34,44,45].

The higher percentage of ANA positivity with higher staining intensity in Rheumatology referrals indicates an optimal utilisation of resources, and patients with ambiguous criteria would benefit from a specialist rheumatology referral within a hospital setting. Routine ANA screening is typically performed at a 1:100 dilution, and rare ANA patterns should be tested at higher dilutions based on clinical suspicion. All rare patterns with a 2+ intensity showed no association with autoimmune disease; therefore, fluorescence intensity can be used as a surrogate marker for antibody titers, and further dilutions can be tested upon the clinician's request. Further prospective studies on the relevance of rare patterns at higher dilutions would be useful based on the findings of the present study.

Limitation(s)

Due to the retrospective nature of the study, it was only possible to analyse the available documented diagnoses for some rare patterns. The study did not include the analysis of patterns at serial dilutions.

CONCLUSION(S)

Limiting ANA screening to patients with established clinical criteria and a high pretest probability of AARD leads to better utilisation of laboratory services. Patients with unclear criteria would benefit from a specialist Rheumatology referral. The highest ANA positivity was observed among Rheumatology referrals, while the lowest positivity was seen in surgical departments in the present study. A significant proportion of ANA-positive patients from the Rheumatology department displayed a 4+ fluorescence intensity, increasing the likelihood of a possible autoimmune disease. Mitotic ANA patterns are rare, and their clinical relevance depends on the fluorescence intensity and antibody titers.

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