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ORIGINAL ARTICLE

Antinuclear antibody titration and pattern are helpful in the diagnosis of systemic autoimmune rheumatic diseases

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Abstract

Introduction: Anti-nuclear antibody (ANA) testing is among the most common immunological test requested in the diagnostic immunology laboratory. The main purpose of this test is to screen for the underlying systemic autoimmune rheumatic diseases (SARDs). The gold standard laboratory method for ANA detection is by the indirect immunofluorescence (IIF) assay. In most laboratories, positive ANA-IIF is reported in terms of titration and pattern. *Objective:* This study was conducted with the aim of determining the correlation between ANA-IIF titration and pattern for the diagnosis of SARDs. *Materials and methods:* A retrospective study was conducted whereby the positive ANA-IIF samples from 1st July 2018 until 31st December 2019 and 1st January 2021 until 31st March 2021 were included in this study. The duplicate samples were excluded. ANA-IIF titration and pattern were recorded for all patients. The demographic, clinical, and final diagnosis data were retrieved from each patient's clinical note. *Results:* A total of 179 patients were included for analysis. The majority of the patients were female (79.9%) and from Malay ethnicity (66.5%). Sixty-five patients (36.3%) had ANA-IIF positive at 1:80 titration followed by 45 patients (25.1%) positive at titration of equal or more than 1:160. Speckled was the predominant pattern visualised in 90 patients (50.3%) followed by homogeneous in 76 patients (42.5%). Forty-five patients (25.1%) were finally diagnosed with SARDs with 41 of them diagnosed as SLE. ANA titration was significantly associated with the final diagnosis of SARDs at all titres ($p<0.001$) but the best cut-off was noted at a titre of equal or more than 1:320 with the sensitivity and specificity of 86.7% and 77.6% respectively. The homogeneous pattern was also significantly associated with SARDs ($p=0.04$). The final diagnosis of SARDs were significantly higher in female ($p=0.03$) and their age was significantly younger ($p<0.001$). *Conclusion:* ANA-IIF titration of equal or more than 1:320 can be used as the best titration for differentiating between SARDs and non-SARDs in a positive ANA sample. Patients with homogeneous pattern were more likely to be diagnosed with SARDs than other ANA-IIF patterns.

Keywords: Anti-nuclear antibody, indirect immunofluorescence, pattern, titration, systemic autoimmune rheumatic diseases

INTRODUCTION

Anti-nuclear antibody (ANA) is a group of antibodies directed against antigens in the nucleoplasm of a host with underlying autoimmune disorders. The anti-nuclear antibody test is one of the most commonly requested laboratory tests when underlying autoimmune disease is suspected in an individual patient. This autoantibody is considered as the hallmark of systemic autoimmune rheumatic diseases (SARDs). However, not all of the SARDs patients will have positive ANA. In general, different laboratory methods are available for the detection of ANA. The indirect immunofluorescence (IIF) method is for long considered as the gold standard in the detection of ANA. Most of the time this is performed on the Hep-2 cells. It is believed that this IIF method is the most sensitive method

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for ANA screening as it is essentially able to detect all autoantibodies with variation clinical significance.[1] It was noted that ANA can also be present in those without underlying SARDs, thus limiting its specificity.

It was found that about 12.9% of the healthy individuals had positive ANA.[2] This is perhaps related to the cut-off of ANA-IIF titration. It was demonstrated that at the screening cut-off of 1:100, only 2.1% of the healthy population had positive ANA.[3] Thus, at the higher ANA titration, ANA is perhaps more specific in diagnosing SARDs. In a long duration follow up analysis, it was noted that those with high titre were highly associated with the diagnosis of autoimmune diseases.[4] There are other methods of ANA detection such as by enzyme-linked immunosorbent assay (ELISA), immunoblot, or Western blot but these methods are generally low in sensitivity but higher in specificity for diagnosis or identification of specific autoantibodies. Thus, in the initial screening for underlying SARDs, ANA-IIF is the preferred screening test due to its sensitivity, inexpensive, and easily performed with other laboratory methods for the identification of the specific antibodies that are preferred as the secondary determination tests.[5] Nevertheless, ANA-IIF was superior to the ANA solid-phase in diagnosing certain SARDs such as SLE and systemic sclerosis as the positive rate in those conditions was between 90%-95% and 85%-95% respectively.[6]

The pattern of ANA-IIF may also be very helpful in distinguishing SARDs and non-SARDs. ANA-IIF pattern will help to provide some diagnostic clues for the underlying SARD. For example, among the SLE patients, the most common pattern seen was homogeneous-speckled.[7] The nuclear dense-fine speckled (DFS) pattern, on the other hand, was more apparent in healthy individuals.[2] Although the previous studies had highlighted the role of ANA-IIF titration and pattern in distinguishing SARDs and non-SARDs, it was important to emphasize that SARDs and non-SARDs can be overlapped at all ANA-IIF titres and patterns. This study was conducted to evaluate the value of positive ANA-IIF titration and its association pattern with the diagnosis of SARDs.

MATERIAL AND METHODS

Study design and setting

A retrospective study was conducted whereby 179 patients with positive ANA-IIF results between 1st July 2018 to 31st December 2018 and 1st January 2021 to 31st March 2021 were included. The ANA-IIF was performed in the diagnostic immunology laboratory. We ensured that there was no duplication of samples from the same patient within this study period. The demographic and clinical data of each patient were collected from the Integrated Laboratory System (ILS) and through each patient's clinical note. The final diagnosis of SARDs was retrieved from the clinical note and the diagnosis was made by the attending physician. All patients were age 18 years old and above. This study was reviewed and approved by the Ethical Committee of the Faculty of Medicine, Universiti Kebangsaan Malaysia with approval number, FF-2019-225 and FF-2021-104.

Anti-nuclear antibody by indirect immunofluorescence (ANA-IIF)

In our routine practice, the ANA-IIF was performed as a screening test for ANA. The ANA-IIF was performed on the Hep-2 cells. The initial screening dilution was at 1:80. If the sample was positive at the screening dilution, further dilution will be performed which were 1:160, 1:320, and finally 1:640. We used a commercial ANA-IIF kit which is NOVA lite Hep-2 ANA (INOVA Diagnostic, San Diego, US). This kit used two conjugates; FITC (fluorescein isothiocyanate) and DAPI (4',6-diamidino 2-phenylindole). The test was performed according to the manufacturer protocol. In each run, the positive and negative control were included. The ANA-IIF slide will then read by the automated microscope system, NOVA view instrument (INOVA Diagnostic, San Diego, US). The instrument analysed the ANA-IIF using DAPI and FITC filters. The DAPI was used by the software for the detection of the cells and focusing while the FITC was used for analysing the immunofluorescence pattern. For each well, 3 to 5 images were captured for analysing purposes. The light intensity unit (LIU) of more than 48 was considered as positive. This is the cut-off set by the manufacturer. The system can determine homogeneous, speckled, nucleolar, centromere, dots, and cytoplasmic patterns. For each reading, two independent readers will determine the pattern and titration. When there was disagreement between the two readers, the third reader will be called to help with the determination of the ANA-IIF pattern. All the readers had more than 10 years of experience in reading ANA-IIF and 5 years of experience in operating the NOVA-view system.

Statistical analysis

The final diagnosis of each patient was classified into SARDs or non-SARDs. The titration was classified according to the titration performed, namely, 1:80,1:160, 1:320, and 1:640. This was further classified into different dichotomous data; equal or more than 1:160, equal or more than 1:320, and at 1:640. For each dichotomous analysis, the chi-square analysis was performed with the final diagnosis of SARDs or non-SARDs. The sensitivity, specificity, positive, and negative predictive value was also calculated. For ANA-IIF pattern, the results were categorized into homogeneous and non-homogeneous patterns. This was again analysed by the chi-square analysis. The continuous variable that was analysed with SARDs or non-SARDs the patient's age. The independent t-test was performed whereby the mean age between the two groups was compared. For all analyses, the two-tailed $p < 0.05$ was considered as significant. The statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) software version 27.

RESULTS

Demographic

One hundred and seventy-nine patients with positive ANA-IIF were included for analysis. This consisted of 36 males and 143 females. The mean age of the patients was 51.85 years. The mean age of female patients was significantly lower than male patients, 50.41 years versus 57.56 years (p=0.025). The majority of the patients were 119 (66.5%) Malay followed by 44 (24.6%) Chinese and 12 (6.7%) Indian. Forty-five (25.1%) patients were finally diagnosed with SARDs and majority of them were female. Most of the patients with SARDs were diagnosed with systemic lupus erythematosus (SLE) (N=41, 91.1%). The distribution of ANA-IIF titration and pattern was as shown in Table 1.

Correlation between the demographic of the patients with the diagnosis of SARDs

As shown in Table 2, there was a significant correlation between gender and age with the final diagnosis of SARDs. The diagnosis of SARDs was significantly diagnosed among female patients and those with SARDs were significantly younger.

TABLE 1: The baseline demographic, anti-nuclear antibody titration and pattern

Parameter		Number	Percentage
Gender	Male	26	20.1
	Female	143	79.9
Ethnicity	Malay	119	66.5
	Chinese	44	24.6
	Indian	12	6.7
	Others	4	2.2
ANA titration	1:80	65	36.3
	1:160	45	25.1
	1:320	25	14.0
	1:640	44	24.6
ANA pattern	Homogeneous	76	42.5
	Speckled	90	50.3
	Nucleolar	3	1.7
	Centromere	6	3.4
	Cytoplasmic	2	1.1
	Others	2	1.1
Final Diagnosis	SARDs	45	25.1
	Non-SARDs	134	74.9

TABLE 2: The demographic correlation with diagnosis of SARDs

Parameter		SARDs		*p*-value
		Yes	No	
Gender	Male	4	32	0.03*
	Female	41	102	
Ethnicity	Malay	31	88	0.581*
	Chinese	10	34	
	Indian	2	10	
	Others	2	2	
Age	Mean	41.24	55.41	<0.001^
	Standard deviation	15.75	16.08	

*data was analysed by the chi-square analysis.
^data was analysed by the independent t-test.

The correlation of ANA-IIF titration and pattern with diagnosis of SARDs

All groups of ANA-IIF titration were found to be significantly correlated with the diagnosis of SARDs but at the highest odds ratio was found in the group of titrations between 1:320 to 1:640. The sensitivity of ANA was in decreasing order which was 93.3% to 66.7% for the titration of between 1:160 to 1:640. The specificity, on the other hand, was in increasing order for the titration between 1:160 to 1:640 with the specificity of 46.3% to 89.6% as shown in Table 3.

The titration cut-off at 1:320 was found to

TABLE 3: The correlation of ANA-IIF titration and pattern for the diagnosis of SARDs

Parameter		SARDs		p-value	Odd Ratio (OR)	Sensitivity, Specificity. Positive (PPV) and Negative Predictive Value (NPV)
		Yes	No			
1. Titration						
a. ⩾1:160	Yes	42	72	<0.001	12.05 95% CI (3.56-40.82)	Sensitivity: 93.3% Specificity: 46.3% PPV:36.8% NPV:95.4%
	No	3	62			
b. ⩾1:320	Yes	39	31	<0.001	21.6 95% CI (8.36-55.77)	Sensitivity: 86.7% Specificity: 77.6% PPV: 55.7%
	No	6	103			
c. 1:640	Yes	30	14	<0.001	17.14 95% CI (7.47-39.35)	Sensitivity: 66.7% Specificity: 89.6% PPV: 68.2% NPV: 88.9%
	No	15	120			
2. Pattern	Homo-geneous	25	51	0.04	2.03 95% CI (1.03-4.03)	Sensitivity: 55.6% Specificity:61.9 % PPV: 32.9% NPV: 80.6%
	Non-homo-geneous	20	83			

have the optimal cut-off to differentiate between SARDs and non-SARDs. It has good sensitivity and specificity with the highest odd ratio. The area under the curve (AUC) at this point showed a good correlation with the value of 0.856 as shown in Figure 1. In Table 3, the homogeneous pattern was significantly associated with the diagnosis of SARDs with p=0.04 with the OR of 2.034.

DISCUSSION

We showed that among the patients with positive ANA-IIF, ANA-IIF titration, and pattern were significantly associated with the diagnosis of SARDs. We noticed that the ANA-IIF titration at 1:320 or more and homogeneous pattern would be able to discriminate between SARDs and non-SARDs. The patients with SARDs in this study were significantly diagnosed at a younger age compared to those without the disease and majority of them were female.

Gender and age play important roles in the development of SARDs. Most of the autoimmune diseases were diagnosed in females rather than males. This is perhaps related to the hormonal factor whereby estrogen plays an important role, particularly in SLE. In SLE, a clear correlation was established between the high estrogen levels and an increase in the number of autoreactive B cells.[8] Many previous studies also described that ANA positive was more common among female rather than male patients. Li *et al*. for example described ANA levels which were measured by ELISA was higher in female than male.[9] Similarly, another report showed a higher proportion of female patients were found to have high ANA titre of equal or more than 1:1280.[4] In contrary, Abeles & Abeles showed no gender difference in term of SARDs diagnosis although the majority of their patients with positive ANA was female.[10] We found that our patients with SARDs were significantly younger than those without the disease. This was contradicted to the previous report in which the age group between 51 to 66 years old was the predominant age group of ANA positive.[2] Another study showed that age was not significantly associated with ANA levels.[9] We think that the younger age group for SARDs was over-represented by the fact that most of the SARDs patients were diagnosed with

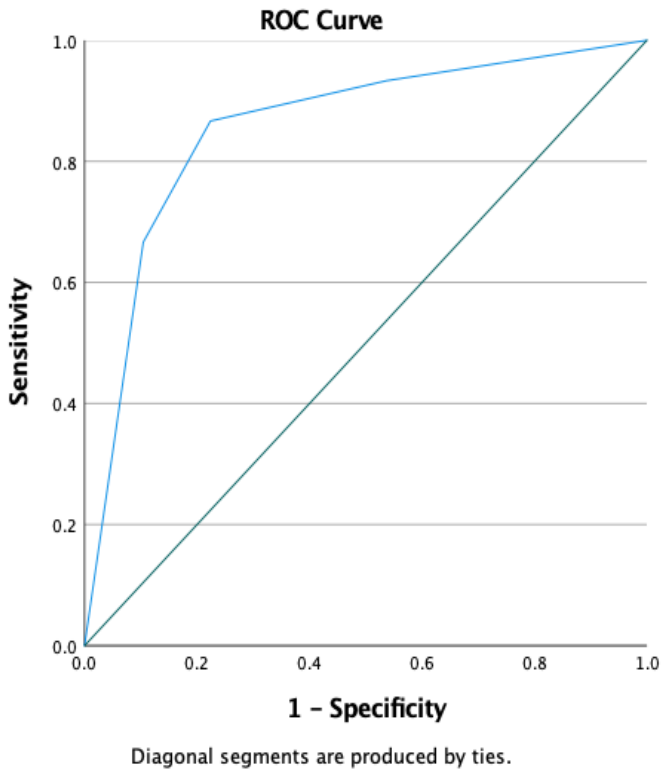

FIG. 1: The Receiving Operating Curve for the ANA-IIF titration and the diagnosis of SARDs. The area under the curve was 0.856. The cut off titration of 1:320 had the sensitivity of 0.867 and 1-specificity of 0.224.

SLE. It was noted that patient with SLE was commonly diagnosed at a younger age and in one study it was found the average age for SLE diagnosis was at 45 years which was younger than those with rheumatoid arthritis and mixed connective tissue diseases with an average age of 50 years.[11]

The evidence of ANA-IIF titration related to the diagnosis of SARDs was not conclusive. In our population, we showed that the best titration to differentiate between SARDs and non-SARDs started from titration of 1:320 and more. However, the previous study showed that the diagnosis of systemic rheumatic disease was commonly diagnosed at dilutions of over 1:320.[12] Similarly, another result described that autoimmune disease was more likely to be diagnosed when the ANA-IIF titration was equal to or more than 1:640.[4] Another study showed ANA-IIF titration was skewed towards high titre among those with SARDs.[2] There was also a significant association found between ANA-IIF titration and the diagnosis of autoimmune diseases in patients with positive both ANA and anti-extractable nuclear antigen (ENA) antibody.[13] Thus, the diagnosis of SARDs is more likely to be made when the ANA-IIF is positive at high titration. However, there was also a report that showed there was no difference in titration to differentiate between SARDs and non-SARDs even at titration of equal or more than 1:640.[14] We also found that 31 of our patients with positive ANA-IIF at titration of equal or more than 1:320 were not diagnosed with SARDs. Thus, it is important to take into account that those without SARDs can even have positive ANA at 1:640. However, the proportion was low in comparison to those with SARDs (13.8% versus 77.8%). This was reflected in the previous study whereby the proportion of those with the disease was higher at titration of over 1:320, 57% with the disease versus 26% without the disease.[12] Interestingly, SARDs was unlikely to be diagnosed at the lower titration. We demonstrated that none of our SARDs patients was diagnosed at titration of lower than 1:160. This finding concurred with the previous report.[10]

The performance of ANA-IIF testing in terms of sensitivity, specificity, positive, and negative predictive value changed according to different titration. We demonstrated the sensitivity was inversely changed concerning the titration whereby the specificity increased when the titration was higher. We showed that the sensitivity reduces from titration1:80 to 1:640 while the specificity increases from titration 1:80 to 1:640. Accordingly, the previous study showed that at the screening dilution of 1:80 the sensitivity and specificity were 90.2% and 87.1% respectively but at the dilution of 1:5120, the sensitivity and specificity changed to 44.4% and 99.2% respectively.[2] They also reported that the ANA-Hep2 test had a good performance in differentiating patients with autoimmune rheumatic diseases with those healthy individuals.[2]

Different ANA-IIF pattern is associated with certain underlying systemic rheumatic diseases. We found that patients with a homogeneous pattern were more likely to have underlying SARDs. Perhaps this was over representative because most of our SARDs were diagnosed as SLE. Homogeneous ANA-IIF pattern was found more commonly in association with SLE although this was not always the case. One study among the SLE populations in Sweden noted the most common pattern in SLE was homogenous (54%) followed by speckled (22%).[7] However, in another study in India showed that the most common pattern seen among SLE patients was speckled (52.1%) followed by homogeneous (37.4%).[3] Concerning speckled pattern, the coarse rather than dense fine-speckled was found to be associated with the SARDs diagnosis.[2] The dense fine-speckled (DFS) pattern is thought to be more common in a healthy population but this association only valid if the DFS pattern is confirmed as monospecific for DFS70.[15] The majority of patients with positive anti-DFS70 antibody were found not to have underlying SARDs.[16] However, in this study, we did not further classify the speckled pattern into coarse, fine, or dense fine. It is interesting to correlate this pattern with the clinical diagnosis of SARDs among our population. The identification of the DFS pattern remains challenged. In one analysis, the unmixed DFS pattern was recognized with significantly lower accuracy in comparison with other more common patterns such as homogeneous, speckled, and nucleolar. This was particularly worse when the DFS pattern was mixed with other patterns.[17]

The limitation of this study needed to be highlighted. First, we only included those with positive ANA-IIF. Thus, the calculation of the sensitivity, specificity, positive, and negative predictive value was without those with a negative result. However, as we showed that most of our SARDs patients were diagnosed with SLE and most of SLE patients will have

positive ANA, this may not significantly change the sensitivity and specificity. Second, although this was a retrospective study, we only managed to retrieve the follow-up data of the patient at the time of ANA testing was performed. Thus, some of the patients, particularly those with high titration of 1:640 or more maybe later diagnosed with SARDs which was not identified during data collection. It was described previously, those with high titre were significantly diagnosed with autoimmune diseases at 6 months follow up.[4] Persistence ANA-IIF positive at higher titration is more likely to be associated with certain underlying SARDs rather than underlying infection or non-specific inflammation.[5]

CONCLUSION

This study showed that the ANA-IIF titration of 1:320 or more provided the best diagnostic performance for the diagnosis of SARDs and homogeneous pattern was significantly associated with SARDs diagnosis. However, we believed that proper clinical data intake together with ANA-IIF results interpretation are paradigm important for the diagnosis of SARDs as both high titration and specific pattern may also be seen in those without underlying SARDs.

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