RESEARCH ARTICLE

Suggested Rational Considerations for ANA-IF and ENA-Profile Test Requisition: Clinical Manifestation, Gender, Pattern, and Titer of ANA-IF

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Received date: Mar 30, 2023; Revised date: Apr 27, 2023; Accepted date: Apr 28, 2023

Abstract

BACKGROUND: The anti-nuclear antibody immunofluorescence (ANA-IF) test is used for screening of autoantibody presence in patients with suspected autoimmune disease. Positive ANA-IF should be followed-up with extractable nuclear antigens profile (ENA-profile). High ANA-IF sensitivity combined with low ENA-profile sensitivity, and the evolution of ANA-IF requests may result in a higher number of positive ANA-IF but negative ENA-profile. It is necessary to make an objective assessment in determining the conditions in which rational ANA-IF and ENA-profile should be suggested.

METHODS: Data were retrieved retrospectively from the medical records of subjects who performed both ANA-IF and ENA-profile. ANA-IF were examined using immunofluorescence principle with cut-off 1:100. ENA-profile which contained sixteen purified antigens was performed using line-immunoblot principle. Data was analyzed descriptively and analytically using SPSS, and significant result was indicated if $p<0.05$.

RESULTS: The ANA-IF result was dominated by negative (44.9%) and positive-speckled, titer 1:100 (32.9%). Of 923 subjects with positive ANA-IF, 45.4% had a negative ENA-profile. Of 751 subjects with negative ANA-IF, 10.2% had positive ENA-profile. In subjects whose specific clinical entity, the ANA-IF sensitivity and negative predictive value (NPV) in detecting ENA-profile were 93.8% and 93.3%, respectively, but the positive predictive value (PPV) was 63.2%. Women with specific autoimmune manifestation accompanied by ANA-IF homogeneous ≥1:100, or centromeres ≥1:100, or speckled ≥1:320 might have been predicted as subsequent positive ENA-profile with area under curve (AUC) of 77.2%, 76.9%, 79.2%, respectively.

CONCLUSION: ANA-IF should only be indicated for those with specific clinical symptoms. For woman with typical symptoms, the presence of positive ANA-IF with homogeneous ≥1:100, or centromeres ≥1:100, or speckled ≥1:320 should be further followed-up by ENA-profile.

KEYWORDS: ANA-IF, ENA-profile, autoimmune, autoantibody

Indones Biomed J. 2023; 15(3): 253-61

Introduction

Anti-nuclear antibody immunofluorescence (ANA-IF) test is mostly used for screening autoantibodies in people suspected of having autoimmune diseases.(1-3) This test is so sensitive that positive result can be detected in healthy people, elderly, inflammation, infection, malignancy, and other conditions. However, higher ANA-IF titers are found more frequently in autoimmune disease. ANA-IF patterns can also help predict other possibilities. For example, nuclear dense fine speckled pattern is not usually associated with autoimmune disease, whereas homogeneous pattern is usually associated with systemic lupus erythematosus (SLE).(1,4-7)

All positive or negative ANA-IF results but with strong suspicion of autoimmune should be continued with an examination to detect specific autoantibodies, for

example with extractable nuclear antigen profile (ENA-profile)/anti-nuclear antibody profile (ANA-profile). The ANA-IF sensitivity is high (89.2%) but its specificity is low (70.9%), while ENA-profile sensitivity is low (8-69%) but its specificity is high (>90%) so the combination of ANA-IF and ENA-profile will be complementary.(4,6,8)

Although a pattern of ANA-IF has been primarily associated with a specific autoantibody and a specific autoimmune disease, it turns out the pattern and specific autoantibody can be found in other autoimmune diseases. This overlapping makes a difficulty in its application. (4) The other difficulty, the presence of technological development and the increasingly widespread spectrum of autoimmune diseases have resulted in ANA-IF test being requested by other specialist or professional healthcare, rather than just by rheumatology or immunology as was previously. As a result of this evolution, the pre-testing probability of ANA-IF, which was initially high, became very low.(6)

High ANA-IF sensitivity and low ENA-profile sensitivity accompanied by a low tendency of pre-test probability tests will result in many positive ANA-IF results, but negative ENA-profile. Currently, many ANA-IF and ENA-profile requests from various physicians in Indonesia had minimal and varied clinical data. However, the results of ANA-IF and ENA-profile examinations in the Indonesian population have not yet been extensively studied. This study aimed to describe the results of ANA-IF and ENA-profile in the Indonesian population as well as the relationship between the two, so that we can predict in which kind of ANA-IF will result in a positive ENA-profile.

Methods

Study Design and Data Collection

This study employed a cross-sectional design. The sample size calculation required at least 303 samples total, with at least 279 samples positive for both ANA-IF and ENA-profile, which were needed to analyze the association between the pattern of ANA-IF and the specific autoantibody in ENA-profile.

Samples were collected retrospectively from the medical records of the patients who completed both the ANA-IF and ENA-profile at Prodia National Reference Laboratory between July – December 2022. Total sampling was carried out. Patients of all ages, both male and female, who underwent both tests at the same time were included in the study.

The data were classified into three groups based on the availability of clinical information. First, the group without clinical information. Second, the group with non-specific autoimmune symptoms or signs, such as white spots on the skin, rash, itching, hair loss, joint pain, stiff, miscarriage, renal disease, being vaccinated, infection, anemia, *in vitro* fertilization program, and so on. Third, the group whose specific clinical information leads to autoimmune, for example, suspected autoimmunity, SLE, lupus nephritis, scleroderma, rheumatoid arthritis, Sjogren's syndrome, polymyositis, etc. Age was classified as ≤15 years old and >15 years old due to the increasing incidence of autoimmune disease with increasing age, with the youngest average onset was at 15 years old.(9)

The ethical of this study has been approved by the Research Ethics Commission from Faculty of Medicine, University of Trisakti (054/KER/FK/I/2023).

ANA-IF

The ANA-IF which used immunoassay principle was carried out using the IIFT:ANA Mosaic 1A EUROPattern reagent (Cat No. 1512-2010-1) (Euroimmun AG, Lubeck, Germany) on the automatic Euroimmun IF Sprinter instrument (Euroimmun AG). The autoantibodies in the sample were attached to the antigen expressed on the human epithelial cell substrate (Hep-2) and primate liver tissue substrate. The attached antibodies were stained with FITC-labelled anti-human antibodies and were visible with a fluorescence microscope. The ANA-IF cut-off was 1:100.(10)

The test was carried out on samples that had been diluted 100 and 1000 times for later used in semiquantitative reporting of positive ANA IF titers of 1:100, 1:320, 1:1000, or >1:1000. The pattern of ANA-IF was read using conventional immunofluorescence microscope and/or EuroPattern computer-aided immunofluorescence microscopy (Euroimmun AG). The pattern was reported according to fluorescence pattern on nuclear, cytoplasmic, or dividing cells using nomenclature as described on the International Consensus on ANA Patterns (ICAP).(11)

ENA-profile

The ENA-profile was performed using EUROline ANA profile 3 plus DFS70 (IgG) reagent (Cat No. 1590-1601-30 G) (Euroimmun AG) on the automatic EUROBlotOne instrument (Euroimmun AG). This assay used line immunoblot assay principle.(12)

Test strips were coated with parallel lines of 16 highly purified antigens, namely nRNP, Sm, SS-A, Ro-52, SS-B, Scl70, PM-Scl, Jo-1, CENP-B, PCNA, dsDNA,

nucleosomes, histones, ribosomal P-protein, AMA-M2, and DFS70. The diluted patient samples were incubated with the immunoblot strips. The specific antibodies in the samples bound to the corresponding antigenic site. A second incubation to detect the bound antibodies was performed using an enzyme conjugate that yield a colour reaction whose intensity was proportional to the level of antibody in the sample.(12)

The colour intensity was evaluated using EuroLine scanner (Euroimmun AG) and then was reported semiquantitative as negative, borderline, positive 1, positive 2, and positive 3. In this study the borderline results were converted as negative because results in the borderline range was evaluated as increased signal, but it was negative.(12)

Statistical Analysis

Data was analyzed descriptively and analytically using SPSS version 26 (IBM Corporation, Armonk, NY, USA). The association between categorical variables was analyzed using Chi-square or Fisher's Exact test. Spearman correlation test was used to analyze the correlation between the titer of ANA-IF and the result of ENA-profile. The area under curve (AUC) to predict the ENA-profile outcome was analyzed using logistic regression analysis and receiver operating characteristic (ROC) curve. Significant result was indicated if $p<0.05$.

Results

The study included 1674 data of subjects who completed both ANA-IF and ENA-profile in July until December 2022. The age range of the subjects was 1-90 years, the average age was 39±16 years old and a female predominance (76.6%). Table 1 showed the characteristics of the research subject.

Out of total 1674 data, there were 751 negative (44.9%) and 923 positive (55.1%) ANA-IF results. Positive ENA-profile was only found in 581 (34.7%) subjects. Positive ANA-IF result was obtained in a greater amount significantly in women (80.7%) than men (19.3%) (p=0.000). A similar result was obtained in the result of ENA-profile (p=0.011).

Positive ANA-IF results were found to be more frequent significantly in subjects aged >15 years old (95.8%) compared to subject aged ≤15 years (p=0.004) (4.2%). The similar result was found in the result of ENA-profile although there was no statistically significant (p=0.662).

There were 863 subjects (93.5%) with a single pattern and 60 patients (6.5%) with multiple patterns. The most common multiple patterns were speckled-cytoplasmic speckled, which was detected in 17 subjects (28.3%), followed by speckled-nucleolar pattern, which was discovered in 13 cases (21.7%). There was no significant association between single or multiple-pattern of ANA-IF and ENA-profile outcome (p=0.094).

When there were multiple patterns, only the first pattern and its titer was statistically analyzed, as shown in Table 2, because the titer of the first pattern was frequently equal to or higher than the titer of the second pattern. The two most common patterns found in the 923 positive ANA-IF were speckled with titer 1:100 of 498 subjects (54.0%) and homogeneous with titer ≥1:320 of 89 subjects (9.6%).

According to the clinical information availability, only 389 subjects have autoimmune-specific clinical information. Positive results were found to be significantly higher in that group, that were 239 (61%) subjects for ANA-IF (p=0.002) and 161 subjects (41%) for ENA-profile (p=0.004), as compared to the groups with unavailable

Table 1. Characteristic of subject.

Characteristic	n (%)
Age (n=1674)	
≤15 years old	95 (5.7%)
>15 years old	1579 (94.3%)
Gender (n=1674)	
Male	391 (23.4%)
Female	1283 (76.6%)
Clinical Information (n=1674)	
Not available	747 (44.6%)
Non-specific	538 (32.1%)
Specific	389 (23.2%)
Positive Results (n=1674)	
ANA-IF	923 (55.1%)
ENA-profile	581 (34.7%)
Positive ANA-IF (n=923)	
Gender:	
Male	178 (19.3%)
Female	745 (80.7%)
Age:	
≤15 years old	39 (4.2%)
>15 years old	884 (95.8%)
Positive ENA-profile (n=581)	
Gender:	
Male	97 (16.7%)
Female	484 (83.3%)
Age:	
≤15 years old	31 (5.3%)
>15 years old	550 (94.7%)

Table 2. Distribution of ANA-IF pattern and its titer (n=923).

ANA-IF Pattern	Titer				Total n (%)
	1:100	1:320	1:1000	>1:1000	
Homogeneous (AC-1)	7	38	13	38	96 (10.4%)
Speckled, DFS70 (AC-2)	0	4	0	0	4 (0.4%)
Centromeres (AC-3)	0	3	5	9	17 (1.8%)
Speckled (AC-4.5)	498	132	27	71	728 (78.9%)
Nuclear dots (AC-6.7)	1	2	1	0	4 (0.4%)
Nucleolar (AC-8,9,10)	16	13	2	3	34 (3.7%)
Rim (AC-11,12)	0	0	1	2	3 (0.3%)
Cytoplasmic fibrillar (AC-15,16,17)	4	0	0	0	4 (0.4%)
Cytoplasmic speckled (AC-19,20)	19	3	0	3	25 (2.7%)
Cytoplasmic golgi (AC-22)	2	0	0	0	2 (0.2%)
Centrosomes (AC-24)	1	0	0	1	2 (0.2%)
Spindle fibers (AC-25)	1	2	0	0	3 (0.3%)
Chromosomal coat (AC-28)	1	0	0	0	1 (0.1%)
Total	550	197	49	127	923 (100%)

clinical information and non-specific clinical information (Figure 1).

Among all the positive ANA-IF results the most common titer was 1:100 (Figure 2). Titer >1:1000 was found significantly in greater amount in the group with specific manifestation of 63 subjects (26%) than in the group without available data (10%) or the group with non-specific clinical information (9%) (p=0.000).

The positive ENA-profile result showed varied degrees of positivity in overall subjects, namely positive 1 (43.7%), positive 2 (24.8%), and positive 3 (31.5%); as well as in the group with specific clinical information (n=161), namely positive 1 (35%), positive 2 (28.9%), and positive 3 (36.1%). Figure 3 depicted the proportions of the various types of autoantibodies discovered.

The overall data revealed a significant correlation between the ANA-IF and the ENA-profile (p=0.000; OR=10.529; 95% CI: 8.045-13.779) (Table 3), with sensitivity of 86.7%, specificity of 61.7%, positive prediction value (PPV) of 54.6%, and negative prediction value (NPV) of 89.7%.

Sub-analyses also revealed a significant relationship between ANA-IF and ENA-profile in the unavailable clinical information (p=0.000), non-specific clinical information (p=0.000), and specific clinical information (p=0.000) groups. However, the strongest association was found in the specific clinical information group (p=0.000; OR=24.023; 95% CI: 12.009-48.053) with sensitivity of 93.8%, specificity of 61.4%, PPV of 63.2%, and NPV of 93.3%.

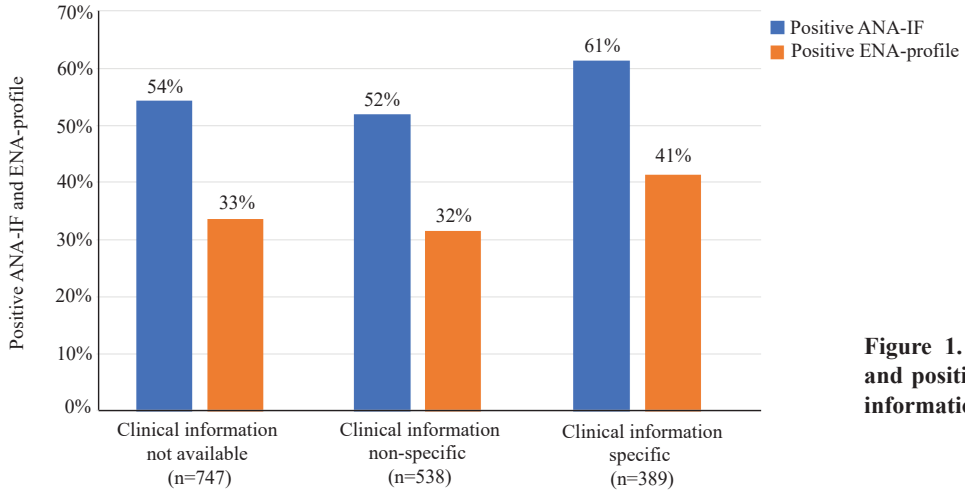

Figure 1. Percentage of positive ANA-IF and positive ENA-profile based on clinical information.

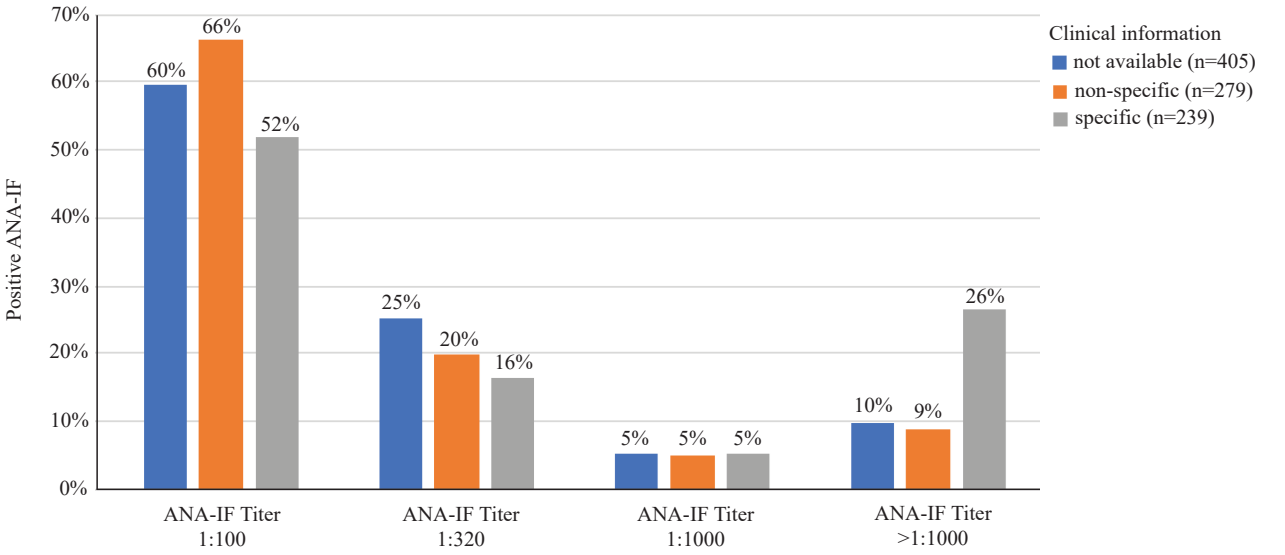

Figure 2. Distribution of ANA-IF titer based on clinical information (total n=923).

There were 419 participants (45.4%) with a negative ENA-profile among the 923 subjects with a positive ANA-IF. The two most common patterns were speckled, which appeared in 342 of the 419 subjects (81.6%) and nucleolar, which appeared in 16 of the 419 subjects (3.8%).

There were 77 participants with a positive ENA-profile (10.2%) among the 751 subjects with negative ANA-IF. In the clinically specific group, 10 subjects with positive ENA-profile were acquired from 150 subjects with negative ANA-IF (6.7%). The specific autoantibodies discovered were vary, but those with a frequency of more than 8% were AMA-M2, histones, Jo-1, Pm-Scl, Scl-70, SSB, and Ro-52, and they often showed grade 1 positive.

The result of this study also showed that there was a significant weak positive correlation between the titer of ANA-IF and the ENA-profile outcome (r=0.380; p=0.000). There was also significant associations between the pattern of ANA-IF and the result of ENA-profile, *i.e.*, homogeneous (p=0.000; OR=2.519; 95% CI: 1.576-4.026), speckled (p=0.000; OR=0.509; 95% CI: 0.360-0.718), and centromeres (p=0.000, OR=1.864; 95% CI: 1.754-1.980). Those patterns showed association with varying types of antigens as listed in Table 4.

Female, particular clinical symptoms, positive ANA-IF, titer of ANA-IF and certain pattern of ANA-IF (homogeneous, speckled, centromeres) were all associated with a positive ENA-profile (p=0.000). The accuracy of prediction to determine the likelihood of a positive ENA-profile was dependent on the cut-off and the pattern of ANA-IF (Table 5).

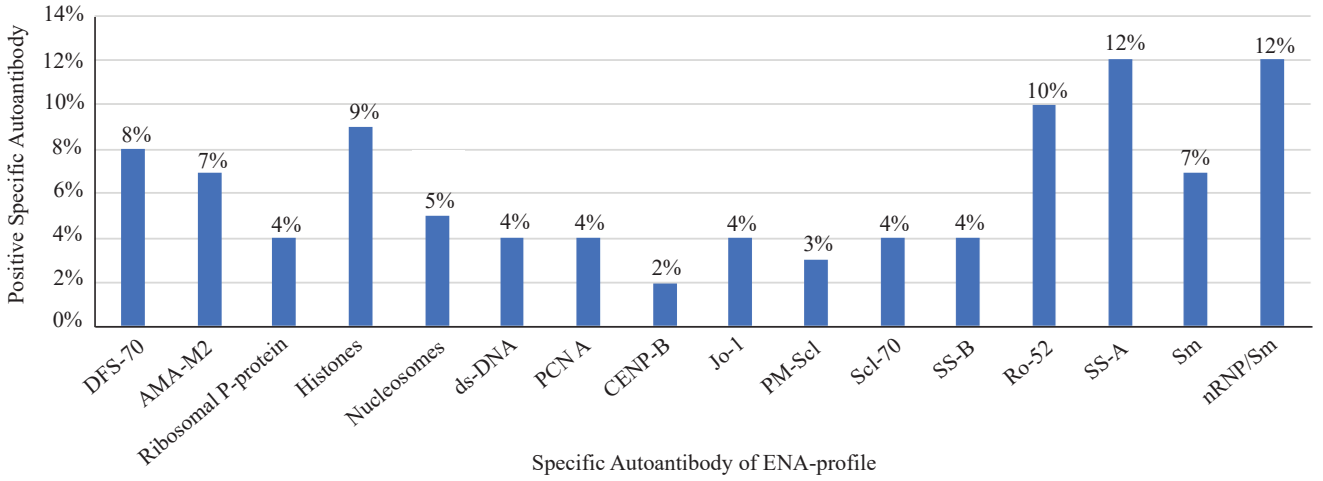

Figure 3. Distribution of specific autoantibody of ENA-profile (n=581).

Table 3. Association between ANA-IF and ENA-profile.

		ENA-profile		Total	*p*- value	OR	95% CI
		Positive	Negative				
All subjects (n=1674)							
ANA-IF	Positive	504	419	923	0.000	10.529	8.045 – 13.779
	Negative	77	674	751			
	Total	581	1093	1674			
Specific Group (n=389)							
ANA-IF	Positive	151	88	239	0.000	24.023	12.009 – 48.053
	Negative	10	140	150			
	Total	161	228	389			

Discussion

Positive ANA-IF was found in 55.1% subjects. This percentage was low in comparison to previous studies, which found positive percentage ranging from 35.1% to 81.2% with an average of 64.5%.(8,13-19) In comparison to ANA-IF, only 34.7% subjects had positive ENA-profile. This was due to the ENA-profile only contained 16 antigens, whilst the ANA-IF contained 100-150 antigens. Furthermore, positive ANA-IF results might be discovered in circumstances unrelated to autoimmune disorders, then it will increase the number of positive ANA-IF.(6,20)

The ANA-IF and ENA-profile in current study were detected primarily in females and subjects aged >15 years old. This is related to the use of the tests for detecting autoimmune disease where the number of the disease is indeed more common in women (80%) and the number increases with age.(9,20,21)

Single or multiple type of autoantibodies which reacted with various antigens in the substrate of ANA-IF would yield single or multiple pattern.(20) In this study the single pattern was predominant (93.5%). Speckled and homogeneous were the two most frequent patterns found. Speckled might present in a broad spectrum of autoimmune diseases, particularly Sjogren's syndrome, SLE, subacute cutaneous lupus, primary biliary cirrhosis, polymyositis, rheumatoid arthritis, neonatal lupus, mixed connective tissue disease, and scleroderma-myositis overlap syndrome. Homogeneous might be found in patients with SLE, chronic autoimmune hepatitis, and juvenile idiopathic arthritis.(22) Both patterns were also found as the most frequent patterns in other studies.(14,15,17-19) Multiple patterns that reflect the presence of multiple autoantibodies was found in only 6.5% in this study. Multiple autoantibodies could be present in multiple/ overlap syndrome of autoimmune.(23)

The ANA-IF results were dominated by negative (44.9%) and positive with titer of 1:100 (32.9%). It was likely that most of the population in this study had a low pretest probability. This is consistent with the literature, which showed that low titer ANA-IF (1:80) was more common in healthy people than in SLE patients, and high titer ANA-IF (1:640) was found 19 times more frequently in SLE patients than in healthy people.(20) This notion was corroborated by the predominate pattern discovered in this investigation, that was a speckled with a titer of 1:100 in 54.0% subjects. Previous study discovered speckled pattern was found in 50.5% of healthy persons with low titers (1:160).(24)

The clinical information of the research individuals was restricted and varied in this investigation. Although only 23.2% of the study participants were clinically specific,

Table 4. The association between ANA-IF pattern and specific autoantibody in ENA-profile.

Pattern	Specific Autoantibody	*p*- value
Homogeneous (AC-1)	Scl70	0.014
	CENP-B	0.038
	ds-DNA	0.000
	Nucleosomes	0.000
	Histones	0.001
	Ribosomal P-Protein	0.002
	DFS70	0.008
Speckled (AC-4,5)	CENP-B	0.000
	PCNA	0.022
	ds-DNA	0.002
	Nucleosomes	0.000
	Histones	0.008
Centromeres (AC-3)	CENP-B	0.000

Table 5. The AUC of several ANA-IF cut-off to predict a positive ENA-profile.

Female, Specific Clinical Manifestation, and Positive ANA-IF		AUC	95% CI	*p*-value
Cut-off	Pattern			
1:100	Homogeneous	77.2	74.9 – 79.5	0.000
1:320	Homogeneous	71.9	69.2 – 74.6	0.000
1:100	Speckled	77.3	75.0 – 79.6	0.000
1:320	Speckled	79.2	76.9 – 81.5	0.000
1:100	Centromeres	76.9	74.6 – 79.2	0.000
1:320	Centromeres	72.1	69.4 – 74.9	0.000

the number of positive ANA-IF and ENA-profile and the titers >1:1000 were significantly more frequent found in this clinically specific group. In fact, the overall prevalence of positive ANA-IF is around 25%, but only 2.5% have substantially high titers. Due to the high prevalence of positive in the general population, requests for ANA-IF tests should be directed more specifically to individuals who have a strong suspicion of autoimmune disease.(20) If the ANA-IF findings are positive or negative but with strong clinical suspicions, the ANA-IF should be followed up with ENA-profile to detect specific autoantibodies.(6)

Positive ENA-profile results revealed varying degrees of positivity with ≥10% autoantibodies identified in nRNP/Sm, SS-A, and Ro-52. The same type of dominant specific autoantibodies was also found in a study in Pakistan population.(17) Specific autoantibodies can help lead to certain autoimmune diseases. Anti-RNPs-Sm is found in 95% of mixed connective tissue disease. anti-SS-A and anti-Ro-52 were found in 40-95% and 70-90% respectively in Sjogren's syndrome.(12)

There was a significant relationship between the results of ANA-IF and ENA-profile with the strongest relationship was in the clinically specific group (p=0.000; OR=24.023, 95% CI: 12.009-48.053). The ANA-IF positive could capture up to 93.8% of the findings with a positive ENA-profile in a clinically specific population, albeit it should be noted that if the ANA-IF result was positive, the ENA-profile had only a 63.2% chance of being positive. On the other hand, if a negative ANA-IF result was discovered, the ENA-profile was 93.3% likely to be true negative. Because a negative ANA-IF appeared to be more effective in excluding the possibility of a positive ENA-profile, this study proposed that the ENA-profile be offered specifically to subjects with positive ANA-IF, as described in literatures.(6,20)

There were 45.4% subjects with negative ENA-profile among 923 subjects with positive ANA-IF and the two most common patterns found were speckled (81.6%) and nucleolar (3.8%). The finding of positive ANA-IF but negative ENA-profile may occur because the type of antigen available on the ENA-profile is not as complete as in ANA-IF.(6) For example, Mi-2, TIF1-β, TIF1-γ, Ku, RNA polymerase III are antigens that also associate with speckled pattern, but these antigens are not available in the current used ENA-profile.(11) Furthermore, false positive ANA-IF can be due to non-specific antibody adsorption and some antigens have similar epitopes so that cross reactions can occur.(20) Similar results were discovered in the previous study, in which 16 out of 110 individuals (14.5%) had positive ANA-IF but negative ENA-profile, with the main patterns being speckled (50%) and nucleolar (37.5%).(18)

There were 10.2% subjects with positive ENA-profile which showed diverse specific autoantibodies (AMA-M2, histones, Jo-1, Pm-Scl, Scl-70, SSB, Ro- 52) among the 751 subjects with negative ANA-IF. In a previous study, 14.8% of cases had negative ANA-IF but positive ENA-profile with diverse kinds of autoantibodies, the most frequently found being Jo-1 and SS-A (Ro60).(14) Despite the fact that ANA-IF is sensitive, false negative ANA-IF can be found, particularly for SSA (Ro60), Ro52, ribosomal P protein, and Jo-1. This might due to these antigens are only found in trace concentrations in substrate cells of ANA-IF. Antigen denaturation during ANA-IF substrate preparation or fixation might also result in false negative ANA-IF. (6,16,20,25)

There was a weak significant positive correlation between ENA-profile with the ANA-IF titer and pattern, namely homogeneous, speckled, and centromeres patterns (p=0.000). These three patterns showed a significant relationship with various types of antigens, except centromeres only with CENP-B where positive CENP-B is found in 80-95% of cases of limited cutaneous systemic sclerosis.(11,12) Although a specific pattern or type of autoantibody has been associated with a type of autoimmune

disease, it turns out that the pattern or specific autoantibody can be found in other types of autoimmune disease.(4,11)

Due to the limited information available, further clinical analysis between both the patterns of ANA-IF and the types of autoantibodies with the patient's clinical status was not performed in this study. Pattern and type of specific autoantibodies have been associated with certain clinical or outcomes. For example, in juvenile SLE patients with a fine-coarse speckled more often showed leukopenia, thrombocytopenia, and kidney involvement if the ENA-profile was positive for RNP, Sm, SS-A; whereas it more often showed serositis and hemolytic anemia if the ENA-profile was positive for Ro-52 for that same pattern.(26)

Female, specific clinical presentation, positive ANA-IF results with higher titers and specific patterns (homogeneous, speckled, and centromeres) were found to have a significant association with the likelihood of positive ENA-profile. The accuracy prediction of a positive ENA-profile was higher when using the ANA-IF cut-off of 1:100 for both homogenous patterns (77.1%) and centromeres (76.9%), whereas a higher cut-off of 1:320 was necessary for the speckled pattern (79.2%). This was consistent with the previous study, which stated that for speckled patterns, the titer should be greater than 1:160, while other patterns can be regarded positive at lower levels.(24)

Conclusion

The ANA-IF examination should only be indicated for people with specific clinical symptoms. For woman with typical clinical symptoms, the presence of positive ANA-IF with specific pattern and titer, namely homogeneous ≥1:100, or centromeres ≥1:100, or speckled ≥1:320 should be further followed-up by ENA-profile.

Acknowledgments

The authors thank PT. Prodia Widyahusada Tbk for giving permission and providing the data needed for this research.

Authors Contribution

YS and USI were involved together in research conception, data analysis, result interpretation, manuscript preparation, figure and table design, critical revision of the manuscript. Data acquisition was obtained by YS.

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