

Association between antinuclear antibodies (ANA) patterns and extractable nuclear antigens (ENA) in HEp-2 cells in patients with autoimmune diseases in Riyadh, Saudi Arabia

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SUMMARY Antinuclear antibodies (ANA) and extractable nuclear antigens (ENA) are instrumental biomarkers crucial in the detection of autoimmune disorders (AID) such as systemic lupus erythematosus (SLE), Sjogren syndrome, *etc.*. In the present study, an assessment of the most frequent ANA patterns associated with most detectable ENA that could be used as efficient prognostic markers in the diagnosis of autoimmune diseases was conducted. Data was retrospectively analyzed from AID patients, retrieved from the medical records of King Fahad Medical City, Riyadh, KSA, from January 2016 to October 2018 who underwent ANA immunofluorescence of HEp-2 cells and their ENA detection was studied. Of the 453 total patients, 39/55 AID males (71%) and 332/398 AID females (83.4%) exhibited ANA positivity. The most common pattern was speckled S-ANA (32.4%) in females and homogenous H-ANA pattern (25.4%) in males. The histones were found at higher frequency in different ANA patterns. anti-Sjogren syndrome related antigen A (SSA), anti-ribonucleoprotein antibody (RNP-Sm), and histones were observed to be associated with homogenous and speckled nuclear patterns. Frequencies of ENA in all ANA patterns were found significant at $p < 0.05$ in males and $p < 0.001$ in females. Spearman's rank correlation of ENA within and among the ANA patterns was non-significant. SSA was significantly correlated with RNP-Sm and Sm at $p < 0.05$ and $p < 0.01$, respectively. The extractable nuclear antigens SSA, RNP-Sm, and histones were found associated with the S-ANA and H-ANA patterns. These correlations are of relevance for the accurate diagnosis of autoimmune diseases.

Keywords autoimmune diseases, extractable nuclear antigens, ANA immunofluorescence

1. Introduction

Research shows that autoimmune diseases (AIDs) are usually characterized by the auto-aggression of the body's immune system. Mainly, this is often against the self-antigens through the production of antibodies (1). Ideally, all AID comprises a strange etiology though it is clear that they share a fundamental mechanism in inflammation and destruction of a person's immunity. While at this, it suffices to observe that the antinuclear antibodies (ANA) consist of specific antibodies whose attack is directed at self-proteins in the cellular nucleus possessing features such as small nuclear ribonucleoproteins (snRNP) or deoxyribonucleic acid (DNA). It is observed that indirect immunofluorescence

(IF), through human laryngeal carcinoma HEp-2 cells, is the prevalent technique detecting ANA (2). Essentially, this detection is crucial in the diagnostic process for systemic autoimmune rheumatic diseases (SARD). When positive results are observed, anti-ENA tests follow as a means of confirming the diagnosis. Ideally, the ANA are critical biomarkers during diagnosis for SARDs such as systemic sclerosis (SSc), Sjogren syndrome (SS), polymyositis/dermatomyositis, and mixed connective tissue disease (MCTD) (3,4).

Fundamentally, the ANA assay is vital in detection of a range of antibodies notable in their reaction with antigens within the nucleus and the nucleolus. Apart from these, few ANA are targeted to antigens in the cytoplasm, mitotic cellular apparatus, *etc.* (5).

Susceptible targets of ANA include histone proteins, ds-DNA, DNA/histone complexes (nucleosomes), various nuclear enzymes, other proteins, and RNPs. ANA plays a significant role as diagnostic and prognostic biomarkers or for monitoring of autoimmune diseases. ANA are present in various infectious, inflammatory, and neoplastic diseases, and low levels of ANA are detected in 30% of healthy individuals (6). Thus, this correlation between ANA and specific diseases is indicative of its usefulness in screening specifically as a prognostic marker, and eventually, providing crucial data on a disease's mechanism. Among the antinuclear staining patterns studied are, the speckled, centromere, homogenous, and the nucleolar patterns. Based on the intranuclear distributions of the antigen, the IF-ANA patterns are often subdivided into, nucleolar-(N-ANA), homogenous/chromosomal-(H-ANA), centromere-(C-ANA), speckled/extrachromosomal (S-ANA), nuclear dot, nuclear membrane patterns and mixed patterns (7). Association of H-ANA with normal cells or with AID is controversial.

Analysis of ENA assists in the diagnosis of the exact autoimmune disease. ANA positivity and its clinical significance in autoimmune diseases has been studied worldwide. As the ANA tests facilitate the determination of the presence or absence of autoantibodies, ENA profiling is crucial in the evaluation of the proteins within the nucleus recognized by the antibodies. Additionally, profiling is vital in the analysis of the progression of autoimmune diseases. There is no clear consensus on the prevalence of AID in Saudi Arabia, as the epidemiology of AID is not accurately documented in this region. For SLE, however, there are significant studies though few and inadequate. As such, it is crucial to conduct further research on the disease to accurately determine its epidemiology. In the above prospective, the present study reviewed data of AID patients tested at the Immunology Department, King Fahad Medical City, Riyadh, Kingdom of Saudi Arabia.

The study aim to investigate: *i*) the most prevalent form of IF-ANA staining pattern among AID patients; *ii*) evaluation of the correlation between IF patterns of ANA on the HEp-2 cells and the ENA frequencies in patients with autoimmune diseases.

2. Methods

This retrospective study was carried out in the Immunology and HLA Laboratory, Pathology and Clinical Laboratory of Medicine, King Fahad Medical City, Riyadh, KSA. Hospital ethics committee approved the study. Mainly, this involved documenting ANA patterns and the conduct of ENA tests among patients diagnosed with a myriad of autoimmune diseases. Of the 666 patients recorded between January 2016 and October 2018, 453 presented non-specific autoimmune diseases while 213 had SLE. Of the 453 patients, 398 were

females and 55 males. For the patients with SLE, ENA profiles were lacking; hence that data was excluded.

2.1. ANA detection by immunofluorescence technique

ANA were directly analyzed by indirect IF microscopy using multispot slides with fixed HEp-2 cells as antigen substrate and fluorescein-isothiocyanate (FITC) conjugated γ -chain specific antihuman IgG as detection antibody (DAKO, Glostrup, Denmark). As per the staining of the nucleus and cytoplasm cells, different patterns were described. These were Homogenous (H-ANA), Speckled (S-ANA), Nucleolar (N-ANA), Cytoplasmic (C-ANA), Centromere (Cen-ANA), Mitochondrial (M-ANA), and some mixed patterns such as Speckled + Cytoplasmic (SC-ANA), Homogenous + Mitochondrial (HM-ANA), Homogenous + Nucleolar (HN-ANA).

2.2. ENA profiling

ENA profiling included detection of extractable nuclear antigens such as RNP; anti-ribonucleoprotein antibody, Sm; anti-Smith, Ro (SSA; anti-Sjogren syndrome related antigen A), La (SSB; anti-Sjogren syndrome-related antigen B), Scl-70; anti Scl 70 antibody, Jo-1; anti Jo1 antibody, CenpB and histones, by immune-enzymatic assays and Western blotting.

2.3. Statistical Analysis

The frequency of ANA patterns and ENA was expressed as a percentage. The non-parametric tests -Wilcoxon signed rank test and Kruskal test were used to check significance between ANA and ENA in both genders followed by Tukey's test. The correlation between ANA and ENA was studied using Spearman's rank correlation. $p < 0.05$ was considered statistically significant.

3. Results

A total of 453 AID patients, consisting of 55 males and 398 females, were assessed for ANA patterns. Of the total number of patients, 71% were ANA positive among males and 83.4% were positive among females. Demographic data of patients with percentages of ANA patterns are represented in Table 1. The highest frequency of participants was in the age group from 21-40 years. The frequency of ANA patterns scored in both genders and microphotographs of ANA immunofluorescence patterns are depicted in Figure 1 and Figure 2. The most prevalent pattern identified in males was H-ANA (31.4%), and S-ANA (32.41%) among females. For both genders, C-ANA was least frequent. As for the females, it was observed that there were varied patterns relative to the males. Mainly, this was inclusive of Homogenous + Mitochondrial (HM-ANA), Cen-ANA, and the

Table 1. Demographic data of the studied population

Total	Males	Females
Number	55	398
Age		
0-20	10	37
21-40	24	188
40-60	13	130
> 60	8	43
N-ANA	8	18
S-ANA	11	129
M-ANA	2	11
H-ANA	14	124
C-ANA	1	6
SC-ANA	3	11
Centromere-ANA	-	12
HN-ANA	-	12
HM-ANA	-	2
Negative	16	66

The different ANA patterns are abbreviated as follows: Nucleolar (N-ANA), Speckled (S-ANA), Mitochondrial (M-ANA), Homogenous (H-ANA), Cytoplasmic (C-ANA), Speckled + Cytoplasmic (SC-ANA), Centromere (Cen-ANA), Homogenous + Nucleolar (HN-ANA), and Homogenous + Mitochondrial (HM-ANA).

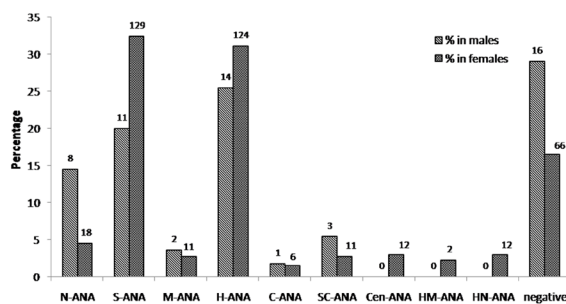


Figure 1. Frequency of antinuclear antibodies (ANA) patterns in male and female AID patients. Data labels represent the frequency (n) positive for the pattern. The different ANA patterns are abbreviated as follows: Nucleolar (N-ANA), Speckled (S-ANA), Mitochondrial (M-ANA), Homogenous (H-ANA), Cytoplasmic (C-ANA), Speckled + Cytoplasmic (SC-ANA), Centromere (Cen-ANA), Homogenous + Nucleolar (HN-ANA), and Homogenous + Mitochondrial (HM-ANA).

Homogenous + Nuclear (HN-ANA). The frequency of different patterns in males and females was compared by Wilcoxon signed rank test (a non-parametric test) that resulted in insignificant differences in the frequencies of ANA patterns in both genders.

Furthermore, to study the most prevalent ENA among the different ANA patterns, we carried out a study of ENA in these patterns, specifically RNP-Sm, Sm, SSA, SSB, Scl-70, Jo-1, Cenp B, and histones. ENA was detectable at different percentages in different patterns. The highest percentage of ENA was observed in the S-ANA pattern. The percentage of ENA in different ANA pattern in males is shown in Figure 3. RNP-Sm, Sm, SSA/Ro52, and SSB were the most frequent ENA identified (about half of the ANA patterns). Scl-70, Jo-1, Cenp B, and histones exhibited altered results. Scl-70 was predominant in H-ANA and N-ANA, Jo-1 was

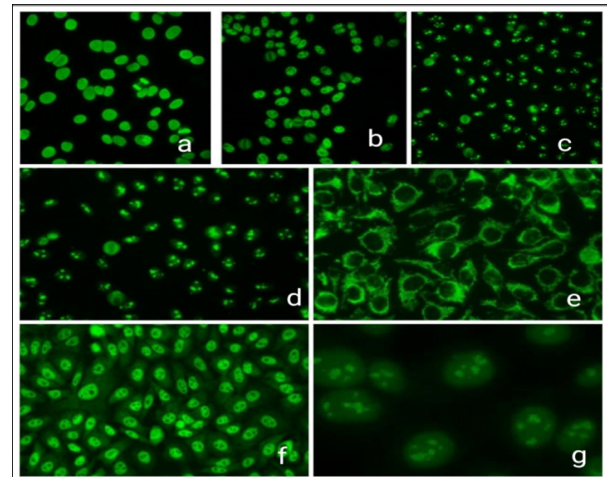


Figure 2. Immunofluorescence patterns in AID patients. (a), Homogenous pattern; (b), Speckled; (c), Nucleolar; (d), Centromere; (e), Mitochondrial; (f), Mixed (Speckled + cytoplasmic); (g), Mixed (Homogenous + Nucleolar).

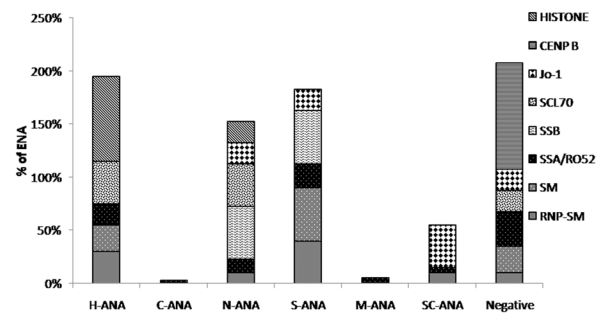


Figure 3. Frequency of extractable nuclear antigens (ENA) in different patterns of antinuclear antibodies (ANA) among males. The different ANA patterns are abbreviated as follows: Homogenous (H-ANA), Speckled (S-ANA), Nucleolar (N-ANA), Cytoplasmic (C-ANA), Centromere (Cen-ANA), Mitochondrial (M-ANA), and some mixed patterns such as Speckled + Cytoplasmic (SC-ANA), Homogenous + Mitochondrial (HM-ANA), Homogenous + Nucleolar (HN-ANA).

predominant in SC-ANA, histones in H-ANA, and Cenp B in a negative pattern. C-ANA and M-ANA were positive only to SSA. In contrast, SSA was detectable in all the ANA patterns. Of the total 55 sera, 16 sera with ANA negative (-) tested positive for ENA. The ENA studied in females yielded similar results. Figure 4 displays the percentage of ENA in different ANA patterns in females. The highest percentage of ENA was observed in S-ANA followed by H-ANA. Of the 398 sera studied in females, 66 sera were ANA negative with ENA positive. SSA positivity was recorded in all ANA patterns, including M-ANA exclusive of SSA. The most prevalent ENA observed was SSA and RNP-Sm compared to all the studied ENA.

To evaluate the inter-relationship between ENA and the correlation between the ENA and the ANA pattern, Spearman's rank correlation was performed. No

significant correlation was observed for both genders by the Kruskal test. However, there was a significant difference in the occurrence of ENA in all ANA patterns at $p < 0.05$ in males and $p < 0.001$ in females. Table 2 shows the interrelationship in the frequency of the ENA in all ANA patterns in both genders. Though different ENA were comparative in the ANA patterns, it suffices to observe that for the males, SSA was considerably correlated to the RNP-Sm and Sm at $p < 0.05$ and $p < 0.01$, respectively. There was significant correlation between Scl-70 and histones ($p < 0.01$) for the females. Similar observations on SSA were observed for Sm and Scl-70 at $p < 0.05$. Scl-70 was strongly correlated with SSA and SSB at $p < 0.05$. A considerable and significant correlation was also seen between Jo-1 /Sm, Jo-1/cenp B, RNP-Sm/Sm, and Jo-1/histone ($p < 0.05$). Essentially, there existed no correlation between ENA within and between the patterns. For a majority of these, it was noted that the ENA was SSA and the RNP-Sm within and between the patterns. Mainly, these were observed to be among the speckled patterns. For the males, ENA

frequency in M-ANA and C-ANA was significantly correlated at $p < 0.001$. Conversely, among the females, there was a notable correlation in the frequency with S-ANA and H-ANA, and S-ANA and N-ANA at $p < 0.01$ and $p < 0.05$, respectively. ENA in C-ANA, also, was significantly related to the ENA in M-ANA at $p < 0.01$. Furthermore, comparison of the ENA within the specific patterns showed significant correlations between RNP-Sm with Sm and SSA with Jo-1 at $p < 0.05$.

It is thus crucial to note that the most frequent ANA patterns identified were homogenous (H) and the speckled (S) patterns. Between the genders, it was observed that $S > H$ for the females and, for the males, $H > S$. In the present findings, the most frequent ENA identified was the RNP-Sm and SSA. Following these were the histones observed to be associated with the H-ANA and the S-ANA patterns. There was a statistical correlation in the frequency of these ENA: SSA with RNP-Sm and Sm; RNP-Sm with Sm in males. In females, the SSA correlated with Sm. The correlation between the most frequent ENAs, SSA and RNP-Sm, was non-significant. SSA was significantly correlated in different ANA patterns at $p < 0.001$ and non-significant in a specific pattern in males. Females showed no significant association between the SSA and ANA patterns. However, RNP-Sm exhibited a significant correlation with all ANA patterns including homologous and speckled ANA. It is thus crucial to observe the analysis of correlation between all ANA patterns.

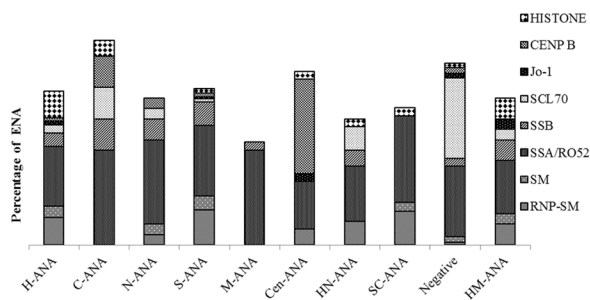


Figure 4. Frequency of extractable nuclear antigens (ENA) in different patterns of antinuclear antibodies (ANA) among females. Homogenous (H-ANA), Speckled (S-ANA), Nucleolar (N-ANA), Cytoplasmic (C-ANA), Centromere (Cen-ANA), Mitochondrial (M-ANA), and some mixed patterns such as Speckled + Cytoplasmic (SC-ANA), Homogenous + Mitochondrial (HM-ANA), Homogenous + Nucleolar (HN-ANA).

4. Discussion

In this study, we found that S-ANA and H-ANA were the most frequent ANA patterns in patients with AID associated with the antigens SSA, RNP-Sm, histones, and SSB. The frequencies of ENA in all ANA patterns were found significant at $p < 0.05$ in males and $p < 0.001$ in females.

Table 2. Correlation between extractable nuclear antigens (ENA) among different ANA pattern in males and females

ENA	SSA	RNP-Sm	Sm	Histone	Scl-70
Males					
RNP-Sm	0.7 (0.05)*	-	0.8 (0.01)*	0.37 (NS)	-
SSA	-	-	0.8 (0.01)*	0.18 (0.66)	-
SSB	0.31 (0.43)	0.49 (0.21)	0.26 (0.54)	-	-
Scl-70	0.46 (0.25)	0.32 (0.43)	0.16 (0.66)	0.87 (0.05)*	-
Jo-1	0.29 (0.4)	0.30 (0.38)	0.06 (0.8)	0.22 (0.60)	-
Cenp B	0.61 (0.12)	0.0 (0.9)	0.34 (0.38)	-	-
Histone	0.18 (0.66)	0.37 (0.38)	0.07 (0.84)	-	-
Females					
RNP-Sm	0.5 (0.07)	-	0.73 (0.01)*	0.42 (0.21)	0.52 (0.10)
SSA	-	-	0.78 (0.05)*	0.59 (0.05)*	-
SSB	0.71 (0.015)	0.5 (0.10)	0.80 (0.002)**	0.58 (0.06)	0.91 (0.0001)***
Scl-70	0.62 (0.04)*	0.52 (0.10)	0.66 (0.033)*	0.55 (0.089)	-
Jo-1	0.46 (0.10)	0.44 (0.1)	0.62 (0.021)*	0.81 (0.001)**	0.62 (0.04)*
Cenp B	0.42 (0.21)	0.15 (0.65)	0.42 (0.2)	0.3 (0.38)	0.38 (0.25)
Histone	0.54 (0.09)	0.42 (0.21)	0.59 (0.05)*	-	0.5 (0.08)

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

The existence of ANA in blood constitutes a significant criterion for the diagnosis of connective tissue diseases (CTD). Identification of ANA subtypes plays a key role in diagnosis of specific CTD. The present study verified the profile of patient samples tested for ENA antibodies and correlated the ENA results with ANA patterns. The most common pattern was speckled S-ANA (32.4%) in females and homologous H-ANA pattern (25.4%) in males. Indeed, females were found to exhibit some mixed patterns apart from the defined ANA pattern compared to males.

Despite ANA specificity to nuclear antigens, cytoplasmic patterns correlated with autoantibodies against cytoplasmic antigens were also observed in some patients. Additionally, some mixed patterns observed could be due to similarity in the epitopes/antigenic determinants of the cellular antigens (8). Among the ENA, SSA/Ro52, RNP-Sm, and histones were found in higher frequency in different ANA patterns. Within the study, SSA was the most prevalent ENA identified, which was found associated with a speckled ANA pattern, around 81.8% in males and 75% in females. The second most was RNP-Sm, with the highest frequency in speckled and homologous patterns. Spearman's rank correlation of ENA within and among the ANA patterns was non-significant. Nevertheless, the frequency of ENA in all ANA patterns was found significant at $p < 0.05$ in males and $p < 0.001$ in females.

A comprehensive understanding on the significance of various patterns assists clinicians in confirming the diagnosis of AID. Notably, the H-ANA pattern has been observed in association with SLE (9). Mainly, the patterns result from the reaction of the antibodies against dsDNA, histones, and DNA/histone complex on HEp-2 cells (10). The S-ANA is released by the antibodies while targeting the extractable nuclear antigens (11). As such, it suffices to observe that the nucleolar pattern results from the antibodies such as RNA polymerase and Scl-70 (topoisomerase-1) (10,12). In a Swedish study by Frodlund *et al.*, H-ANA was reported as the most dominant IF-ANA pattern in patients with SLE (10). The most frequent pattern identified in this study was speckled pattern followed by homologous pattern. The results are in line with a previous study by Peene *et al.* (13).

However, studies of ANA pattern with relevant ENA testing with diagnostic importance are scarce, particularly in the Saudi population. The ENA commonly observed in this study was inclusive of the RNP-Sm and SSA found in association with the speckled pattern. This was in line with the observations by Mutasim *et al.* (14). Similarly, the highest frequency of the anti-SSA/Ro autoantibody in anti-ENA-positive patients was also reported by Lora *et al.* and evidenced by Banhuk *et al.* (15,16). Homologous to the finding of Li *et al.* (17), higher ANA positivity (83.4%) in females compared to males (71%) was observed. Few of the mixed patterns were found in females compared to males. It is possible

that Scl-70, a type of ENA found attached to DNA and extrachromosomally in the nucleoplasm, draws from a mixed IF staining pattern. Similar to Scl-70, the La/SSB antigen may partially localize in nucleoli. Hormonal profiles, and fetal microchimerism, are regarded as potential discriminating factors for such patterns.

As observed, the determination of ENA or anti-ENA profiling contributes to an improved differentiation among the different types of autoimmune rheumatic diseases (ARD). The presence of RNP autoantibodies constitutes an efficient marker in the diagnosis of mixed connective tissue disease (MCTD). Similarly, ANA positivity with dsDNA or Sm positivity forms the criteria for diagnosis of SLE (18). In addition, the anti-SSA/Ro and anti-SSB/La antibodies contribute as a significant immune marker in the detection of Sjogren syndrome, subacute cutaneous SLE, and neonatal lupus syndrome (19). Jo-1; histidyls RNAsynthetase is observed to be capable immunomarker linked with polydermatomyositis. Likewise, the CENP-B and Scl-70 positivity or the appearance of anti-centromere antibodies (CENP-B) or topoisomerase 1 (Scl-70) facilitates the diagnosis of systemic sclerosis (20,21).

Of the many constraints conspicuously displayed with ANA testing, is the ENA positivity displayed in ANA negative samples among patients with AID. Unpredictably, ENA-positivity with ANA-negative patients in 16 AID males (29%) and 66 AID females (16.5%) was noted in the current investigation. These findings are common as these could be cases of patients undergoing immunosuppressive therapy (22). Additionally, immunoassay was observed to be more sensitive for the detection of the SSA/Ro52 relative to ANA-IF for even the Hep-2 cells. Scl-70 and Jo-1 that is associated with systemic sclerosis and polymyositis, and may not be detected in preliminary IF-ANA screening. Mainly, this follows the antibodies' cytoplasmic positivity rather than nucleic staining patterns on IF. Therefore, in such cases, ANA could have been reported, as negative. Negative ANA samples also require further assessment with anti-ENA profiles to facilitate the identification of the relevant ENA associated with the disease in case there is a strong suspicion of AID. Regardless of this, positive results of ANA testing should always be interpreted within the confines of a clinical context.

Additionally, Spearman's rank correlation between different ENA within and between the patterns yielded unsatisfactory results. As such, there was no significant correlation between ENA and ANA. Paramount in the present findings was the significant correlation between different ENA studied. SSA was significantly correlated with RNP-Sm, Sm, and Scl-70. Based on the above associations, it can be postulated that the study comprised a mixed population with different AID such as SLE and Sjogren syndrome. From these perspectives, it can be concluded that IF serves well as a standard ANA testing

method showing high specificity and sensitivity, along with specific ENA detection that assists in diagnosis of autoimmune diseases more accurately, while minimizing cost and time required for conventional immunological evaluation for diagnosis of these diseases. The study, however, had some limitations. Though it confirmed the prevalence of ANA and ENA patterns among patients with autoimmune diseases, the evaluation of IF ANA patterns from specific autoimmune diseases as a comparative study would have added additional information to the outcome of the present investigation.

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