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CLINICAL RESEARCH / KLİNİK ARAŞTIRMA

Comparison of the Results of Indirect Immunofluorescence and Immunoblot in the Detection of Anti-Nuclear Antibodies

Anti-Nükleer Antikorların Tespitinde İndirekt İmmünofloresan ve İmmünoblot Sonuçlarının Karşılaştırılması

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Abstract

Objective: The indirect immunofluorescence (IIF) antibody method using HEp-2 cells for anti-nuclear antibodies (ANA) screening is the gold standard. Some antigens have been purified and termed as extractable anti-nuclear antibodies (ENA) to detect the autoantibodies. These autoantibodies are usually detected by the immunoblot (IB) method. In this study, we compared the ANA patterns detected by the IIF method with the ENA detected by IB to predict which confirmatory test should be selected for which ANA patterns.

Methods: 2894 serum samples sent from different clinics to both ANA and IB between January 2019 and March 2022 were analyzed in the Medical Microbiology Laboratory of University of Health Sciences Turkey İzmir Tepecik Education and Research Hospital.

Results: The ENA positivity rates of samples with positive ANA patterns were centromere 91%, Topo I-like 83%, speckled 66%, homogeneous with other ANA patterns 53%, speckled with other ANA patterns 53%, homogeneous 45%, nucleolar 30%, nuclear dots 28%, nuclear envelope 15%, and dense fine speckled-70 (DFS70) 10%, respectively. In the study, 100 (12.9%) of the clinical specimens with ANA positivity were sent from male patients and 674 (87.1%) from female patients, and the mean age was 43±19.17 years (age range: 0-88).

Conclusion: Our data are highly consistent with the centromere, Topo I-like, and granular patterns in IIF between specific antibody positivity detected in IB for antibodies associated with the pathogenesis of systemic autoimmune rheumatic diseases. We believe that the tests can be used more efficiently with rational laboratory use and can provide more accurate guidance to the clinic.

Keywords: Indirect immunofluorescence antibody, immunoblotting, anti-nuclear antibodies, autoimmune disease

Öz

Amaç: Anti-nükleer antikor (ANA) taraması için HEp-2 hücrelerinin kullanıldığı indirekt immünofloresan (IIF) antikor yöntemi altın standarttır. Bazı antijenler, otoantikorların tespiti için saflaştırılmış ve ekstrakte edilebilir anti-nükleer antikorlar (ENA) olarak adlandırılmıştır. Bu otoantikorlar genellikle immünoblot (IB) yöntemiyle tespit edilir. Bu çalışmada hangi ANA paternleri için hangi doğrulayıcı testin seçilmesi gerektiğini öngörebilmek adına IIF ile tespit edilen ANA paternlerini IB ile tespit edilen ENA ile karşılaştırmayı amaçladık.

Yöntem: Ocak 2019 ile Mart 2022 tarihleri arasında farklı kliniklerden hem ANA hem de IB'ye gönderilen toplam 2894 serum numunesi Sağlık Bilimleri Üniversitesi İzmir Tepecik Eğitim ve Araştırma Hastanesi Tıbbi Mikrobiyoloji Laboratuvarı'nda analiz edildi.

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Öz

Bulgular: Pozitif ANA paterni olan örneklerin ENA pozitiflik oranları sırasıyla: sentromer %91, Topo I-benzeri %83, benekli %66, homojen ile diğer ANA paternleri birlikteliği %53, benekli ile diğer ANA paternleri birlikteliği %53, homojen %45, nükleolar %30, nükleer noktalar %28, nükleer membran %15, yoğun ince benekli (DFS70) %10'dur. Çalışmada ANA pozitifliği olan klinik örneklerin 100'ü (%12,9) erkek hastalar, 674'ü (%87,1) kadın hastalardan gönderilmiş olup, ortalama yaş 43±19,17 yıl (yaş aralığı: 0-88) idi.

Sonuç: Verilerimiz, sistemik otoimmün romatizmal hastalıkların patogenezi ile ilişkili antikorların tespiti için IB'de saptanan spesifik antikor pozitiflikleri ile IIF'deki sentromer, Topo I-benzeri ve granüler paternlerle oldukça uyumludur. Testlerin akılcı laboratuvar kullanımı ile daha verimli kullanılabileceği ve kliniğe daha doğru rehberlik sağlayabileceği düşüncesindeyiz.

Anahtar Kelimeler: İndirekt immünofloresan antikor, immünoblot, anti-nükleer antikorlar, otoimmün hastalık

Introduction

Autoantibodies detected in systemic autoimmune rheumatic diseases (SARD) are usually directed against nuclear antigens and are called anti-nuclear antibodies (ANA). Diseases in which ANAs are positive have a broad spectrum and are referred to as ANA-associated rheumatic diseases[1]. Patterns shown by the indirect immunofluorescence (IIF) method have been shown to be associated with specific diseases, and anti-cell codes (AC) have been established according to the international consensus on ANA models[2]. The IIF method using HEp-2 cells for ANA screening is the gold standard[3]. Patterns shown by the IIF method have been shown to be associated with specific diseases, and AC have been established according to the international consensus on ANA models[2] (classification algorithm and representative images available at www. ANApatterns.org). Nuclear patterns such as homogeneous, speckled, dense fine speckled (DFS) 70, nucleolar, nuclear membrane, centromere, and cytoplasmic and mitotic staining patterns can be reported for ANA. This association between rheumatic diseases and the patterns detected in IIF is due to autoantibodies produced against some specific antigens in the cell. Some of these autoantibodies have been purified and termed as extractable anti-nuclear antibodies (ENA). These antibodies are often detected by enzyme -linked immunosorbent assay (ELISA) or immunoblot (IB) methods. In some clinical situations or depending on the type of HEp-2 cell line used, false negative results may be obtained with the ANA IIF assay. An example of this is the inability to detect isolated Ro-52-positive samples in the IIF. In these cases, screening for antibodies using the anti-ENA profile ELISA or anti-ENA IB is recommended. Conversely, the pattern observed by IFA in HEp-2 cells is not always "confirmed" by the blot, as ENA panels consist of the most observed antibodies. Many antibodies are absent from standard ENA assays[4]. In this study, we compared the ANA patterns detected by the IIF method with the ENA detected by IB to predict which confirmatory test should be selected for which ANA patterns.

Materials and Methods

Patients

Totally 2894 serum samples sent from different clinics to both ANA and IB between January 2019 and March 2022 were analyzed in the medical microbiology laboratory of University of Health Sciences Turkey Izmir Tepecik Education and Research Hospital. The clinical diagnoses of the patients were retrospectively obtained from the medical records.

ANA Screening Using the IIF Method

ANA screening was performed by the IIF method using HEp-20-10 cell substrates (Euroimmun Luebeck, Germany). The assay procedure and evaluation were performed according to the manufacturer's instructions. The screening dilution of the IIF method was 1:100, and values below this dilution were considered negative. Positive samples were identified by two experts based on an international consensus defining ANA patterns (classification algorithm and representative images available at www.ANApatterns.org)[2].

ENA Detection Using the IB Method

ENA (Euroline ANA Profile 1, Euroimmun Luebeck, Germany) detected by the IB method was used according to the manufacturer's instructions. This method was used to determine nRNP/Sm, Sm, SS-A, Ro-52, SS-B, Scl-70, Jo-1, CENP B, dsDNA, nucleosomes, histones, and ribosomal P antibodies.

Statistical Analysis

Statistical analyses were performed using IBM SPSS statistics 21 (SPSS Inc., Chicago, IL, USA) for descriptive and frequency Inc.. The contingency coefficient test was applied to the results. Data are presented as mean ± standard deviation

(SD), number (n) and percentage (%). The p-value <0.05 was considered statistically significant. The study was conducted according to the World Medical Association Declaration of Helsinki for studies on human subjects and was approved by the Scientific Research Ethics Committee of University of Health Sciences Turkey, İzmir Tepecik Education and Research Hospital (dated April 15th, 2022; protocol number: 2022/04-13).

Results

The selected cohort included 2894 sera from children and adults analyzed for routine tests for ANA by IIF and ENA by IB. The distribution of results from 779 ANA-positive samples, 2115 ANA-negative samples, and 604 ENA-positive samples, 2290 ENA-negative samples are shown in Table 1. The IB positivity rates of samples with positive ANA patterns were respectively: Centromere 91%, Topo I-like 83%, speckled 66%, homogeneous + other patterns 53%, speckled + other patterns 53%, homogeneous 45%, nucleolar 30%, nuclear dots 28%, nuclear envelope 15%, and DFS70 10%. The contingency coefficient between the two tests also had a high value (0.72) and was statistically significant (p<0.001). In the study, 105 (13.5%) of the clinical specimens with ANA positivity were sent by male patients and 674 (87.1%) by female patients, and the mean age was 43±19.17 years (age range: 0-88). Age range, mean age, and standard deviation of patterns according to ANA positivity, respectively homogeneous 43.12±16.3 (13-72), DFS70 38.17±14.49 (15-61), centromere 52.85±13.9 (12-75), speckled 45.02± 17.0 (0-88), nuclear dots 56.71±13.9 (2-75), nucleolar 55.75±8.80 (2-73), nuclear envelope 66.33±13.8 (16-82), Topo I-like 51.46±15.7 (6-72) homogeneous + other patterns 32.75±20.4 (7-68), speckled + other patterns 47.56±10.18 (9-66). The female-to-male ratio of the patients was reported as 6.7. The sex of the patients was compared with the ANA patterns according to the ratio of females to total, homogeneous 151/172, DFS70 105/127, centromere 59/61, speckled 215/246, nuclear 24/25, nucleolar 43/54, nuclear envelope 17/20, Topo I-like 12/12, homogeneous+other patterns 34/45, speckled + other patterns 14/17. It was found that 658 (85%) of the patients were in the adult age group (>18 years) and 116 (15%) were in the child age group (≤18 years). A detailed graph comparing IB results with IIF results can be seen in Figure 1, and a detailed graph comparing IIF results with IB results can be seen in Figure 2.

Discussion

ANA detection is the first-line test for laboratory diagnosis of SARD. The IIF method using HEp-2 cells for ANA screening is the gold standard[3]. If the result of ANA is positive, it is recommended that specific tests for anti-ENA antibodies also test for anti-dsDNA antibodies if clinical (Systemic Lupus Erythematosus) SLE is suspected[5]. In cases of strong

Table 1. The distribution of ANA and ENA results

IB Results* / IIF Results	Cent B	SS-A	Ro-52	SS-B	RNP	SCL70	Histon	Nuc	Jo1	Sm	Ribp	Neg	Total	IB pos. %
Homogeneous (AC-1)	5	25	24	6	9	6	26	24	4	2	10	95	172	45
DFS70 (AC-2)	1	2	3	0	1	1	1	1	0	1	1	115	127	10
Centromere (AC-3)	56	4	11	0	3	1	3	1	1	1	0	5	61	91
Speckled (AC-4,5)	3	97	118	49	28	3	13	16	5	21	9	84	246	66
Nuclear dots (AC-6,7)	0	1	5	1	0	0	0	0	2	0	0	18	25	28
Nucleolar (AC-8,9,10)	0	4	4	2	2	1	1	0	2	2	4	38	54	30
Nuclear envelope (AC-11,12)	0	0	3	0	0	0	0	0	0	0	0	17	20	15
Topo I-like (AC-29)	0	2	1	0	0	8	0	0	0	0	0	2	12	83
Homogenous + other paterns	8	8	10	0	4	2	4	2	1	1	2	21	45	53
Speckled + other paterns	0	6	6	3	2	1	0	0	0	0	0	8	17	53
Negative	2	30	75	9	21	31	27	1	28	16	2	1887	2115	11
Total	75	179	260	70	70	54	75	45	43	44	28	2290	2894	21

*The IB reports may contain autoantibody results against multiple antigens, ANA: Anti-nuclear antibodies, ENA: Extractable anti-nuclear antibodies , IB: Immunoblot, IIF: Indirect immunofluorescence, AC: Anti-cell codes

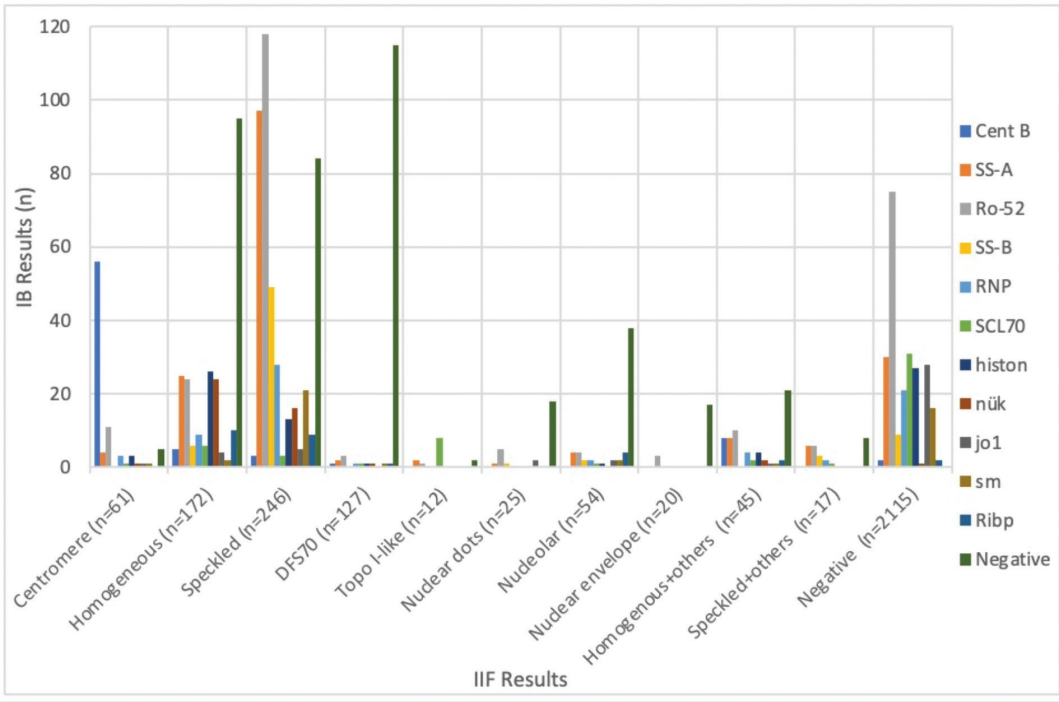

Figure 1. Comparison of the IB and IIF results

IIF: Indirect immunofluorescence, IB: Immunoblot

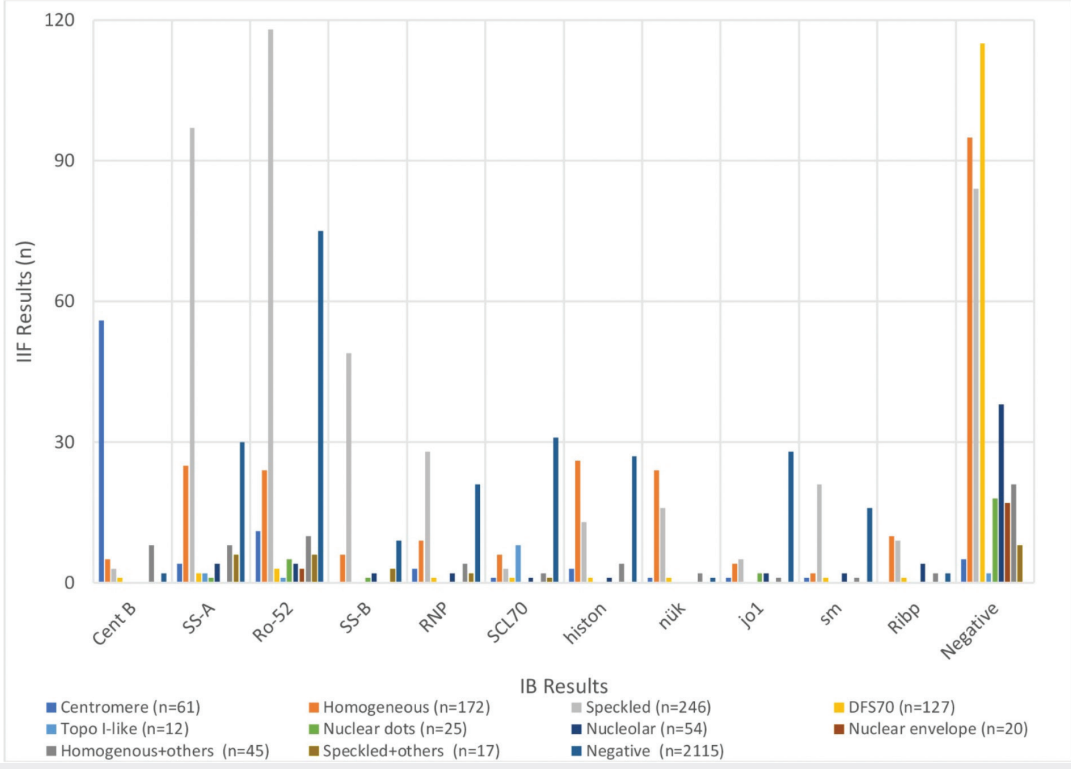

Figure 2. Comparison of the IIF results and IB results

IIF: Indirect immunofluorescence, IB: Immunoblot

clinical suspicion, regardless of the result of the ANA test, the physician's request for the determination of antibodies to specific ENA is accepted[6]. When we compared the results of the ANA test with the results of the IB test in this study, we found differences in the antibodies detected.

One of the frequently detected patterns in the ANA test is the homogeneous pattern. Antigens associated with this pattern are dsDNA, histone, and nucleosome. 45% of the homogeneous patterns detected in our study were positive in the IB test. We think that the most important reason is that we do not detect dsDNA, and DFS70 results in our IB kit. For the determination of anti-dsDNA antibodies, a Farr test with high clinical specificity and Crithidia luciliae IIF test (CLFT) are recommended rather than IB[5]. Alternative methods such as ELISA may have lower specificity. If this is the case, it is recommended that positive results obtained by these methods be confirmed by CLFT or the Farr test and reported separately. Low anti-DFS70 titers and homogeneous + speckled patterns are difficult to distinguish. When we looked, 50 of the 95 reports of homogeneous patterns that were negative by the IB test were low titers. Therefore, we recommend confirming them with the IB test using the DFS70 antigen. We think that the IB distribution of results of samples with homogeneous patterns is different because of the diversity of antibodies, especially in patients with SLE patients. In these patients, dsDNA, nucleosome, and histones are accompanied by many antibodies such as SS-A, Ro-52, SS-B, sm, U1-RNP, and ribosomal P protein[7]. One of the most common patterns when scanning with IIF is the DFS70 staining pattern[8]. Isolated anti-DFS70 antibody positivity is found in less than 5.7% of systemic rheumatic diseases[9,10]. In healthy individuals, these antibodies are frequently found in the serum of healthy individuals[11–13]. In our sample, the third most common pattern was DFS70, but the IB positivity was 10%, similar to the negative IIF results. We attribute these low IB results to the accuracy of the isolated DFS70 positivity in the IIF results and the absence of DFS70 antigen in our IB kit. The centromere pattern, characterized by its morphological appearance, is observed in limited cutaneous systemic sclerosis and Raynaud's phenomenon, and is associated with CENP A, B, C, and F antigens[14]. Although we detected only CENP-B in the IB test, there was 91% positivity. The remaining negative samples can be associated with other antigens. Since there is a high compatibility for this pattern, it might be sufficient to determine the centromere pattern for the IIF test.

The speckled pattern is an important pattern we see in diseases such as mixed connective tissue diseases, SLE, systemic sclerosis, scleroderma[15]. It is particularly associated with SS-A, SS-B, Topo-1, Sm, U1-SnRNP, Mi2, and Ku antigens. Antibodies against SS-A, Ro-52, SS-B in our IB panel are in the majority. We think that the 34% of samples that we detected as IB-negative also had other antibodies that were not in our panel. We see that the IIF-IB match (83%) is even greater for the samples we reported as Topo-1-like. Because the antigens associated with their patterns were not examined in the detection of ENA, there were also false-negative results for the nuclear dots, nucleolar envelope, and nuclear envelope in the study IB. In the IIF assay, there may be problems in detecting anti SS-A, anti-tRNA synthetase (Jo-1), and anti-Rib-P antibodies (anti-Rib-P), depending on the use of the HEp-2 cell line[4]. Because these antigens are expressed at very low levels in HEp-2 cells or may be denatured in tissues during fixation procedures, "false negatives" may occur. If clinical suspicion exists, it is reasonable to request ENA testing even if the IIF result is negative. Ro-52, Topo-I, SS -A, and Jo-1, and histone positivity was observed in the samples that we detected as negative.

Study Limitations

The main limitation of our study was that not all antibodies detected in IIF were present in our IB panel. The IB panel can be expanded to include some of these autoantibodies, such as DFS70. Thus, the results were confirmed by a second immunological method. Or other IB panels can be created for the purpose, such as the Myositis panel. Thus, there may be a greater association with IB for antibodies identified by the IIF method.

Conclusion

Our data are highly consistent with the centromere, Topo-1-like, and speckled patterns in IIF between specific antibody positivity detected in IB for antibodies associated with the pathogenesis of SARD. For samples with homogeneous patterns, dsDNA should be requested as an extra. If patients with a DFS70 pattern do not have systemic rheumatic disease, local rheumatic causes, allergic disease, or a healthy individual may be present, in which case, further testing may not be necessary. Patients should first be screened with IIF for ANA, and if results are positive, specific antibodies should be detected with IB. IB may also be ordered if ANA is negative and there is a strong clinical suspicion. By the rational laboratory use, the tests can be used more efficiently and provide more accurate guidance to the clinic.

Ethics

Ethics Committee Approval: Ethics committee approval was obtained from University of Health Sciences Turkey, İzmir Tepecik Education and Research Hospital Ethics Commitee (decision no: 2022/04-13, date: 15.04.2022).

Informed Consent: Retrospective study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: A.T., Design: A.T., Data Collection or Processing: A.T., Analysis or Interpretation: A.T., N.Y., Literature Search: A.T., Writing: A.T., N.Y.

Conflict of Interest: No conflict of interest was declared by the authors.

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