Research Article

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Evaluation of the performance of immunoblot and immunodot techniques used to identify autoantibodies in patients with autoimmune diseases

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Abstract: Autoimmune diseases are pathological conditions in which the immune system mistakenly attacks its own tissues. This study evaluates the performance of two techniques, which are identifiers of autoantibody specifics: immunoblot and immunodot. This study was conducted in 300 patients of whom 62 were tested positive for antinuclear antibodies. The patients were initially screened for antinuclear antibodies using indirect immunofluorescence. Then, the identification of specific autoantibodies such as anti-extractable nuclear antigens (ENAs) was carried out using the immunoblot and immunodot techniques. The results showed that immunoblot and immunodot did not present a significant difference in their sensitivity against anti-SSA/52, SSB, CENP-B, PCNA, U1-snRNP, Jo-1, Pm-scl, and Mi-2 ($p > 0.05$). However, the two techniques showed a significant difference in their sensitivity toward autoantibodies anti-DNAn, antihistone, anti-SmD1, and anti-ds-DNA ($p < 0.05$). The immunoblot data were in complete accordance with the immunodot data (100%) regarding the detection of autoantibodies such as anti SSA/52, SSB, CENP-B, PCNA, U1-snRP, Jo-1, Pm-scl, and Mi-2, 80% regarding SmD1, and 75% concerning ds-DNA. We should certainly pay closer attention to the efficiency of the techniques used in the diagnosis of autoimmune diseases.

Keywords: systemic autoimmune diseases, antinuclear antibodies, immunoblot, immunodot, indirect immunofluorescence

1 Introduction

Autoimmunity is a pathological state in which the body reacts, through a humoral (autoantibody) and cellular (self-reactive T cells) autoimmunity, against its own healthy cells and tissues [1]. Autoimmune diseases (ADs) affect around 5% of the world's population and are the third leading cause of death in developed countries [2]. Autoimmune diseases are heterogeneous and are usually classified into two groups: specific autoimmune diseases and nonspecific autoimmune diseases. The first type habitually targets antigens located in a specific tissue or a cell, while the second type habitually targets antigens located anywhere in the body. Thus, specific and nonspecific autoimmune diseases are responsible for producing antinuclear antibodies (ANAs).

The antinuclear antibodies (ANAs) are autoantibodies that attack components of cells (anti-DNA, anti-nucleosome, anti-histone, and anti-RNA) triggering autoimmune disorders such as systemic lupus erythematosus [1]. Systemic lupus erythematosus is a nonspecific autoimmune disease that provokes damages in many organs and tissues of the body, such as the skin, heart, kidney, and nervous system. The most common symptoms of systemic lupus erythematosus are severe fatigue, joint pain, headache, hair loss, joint swelling, anemia, blood clotting, and appearance of a rash on the cheeks and nose, which is also known as a "butterfly rash" [3].

Autoantibodies, antibodies designated as SS-A or Ro and SS-B or La, were screened in patients with Sjögren's

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syndrome (SS) and SLE and have been demonstrated to attack intracellular components that may be implicated with adjustment of RNA polymerase III function [4]. Autoantibodies in human sera are commonly identified by immunofluorescence microscopy (IFM). Tissue culture cells or tissue sections have been used as the antigen to identify autoantibodies under the immunofluorescence microscopy system. Even other assays such as enzyme-linked immunosorbent assay (ELISA) and Western blotting (WB) techniques have been used as alternatives to IFM in analyzing substrates; however, these assays will apparently not be a substitute for IFM [5].

The aim of this study is to develop immunological diagnosis methods for the laboratory of Immunology of CHU Morocco to be able to study the antinuclear antibodies released in the blood due to systemic autoimmune diseases. This study is particularly aimed to evaluate the performance of immunoblot and immunodot techniques used to identify specific autoantibodies, specifically the anti-extractable nuclear antigen (ENA).

2 Material and methods

This is a prospective study done for descriptive and analytical purposes. It was conducted at the Immunological laboratory of CHU Morocco, with 300 patients of whom 62 of them were tested positive for ANA. The identification of the ANA was carried out using immunoblot and immunodot assays.

The Human Ethics Review Committee of our University reviewed and approved this study. Furthermore, this study was performed according to the principles outlined in the Declaration of Helsinki, and institutional review boards and ethics committees at participating sites approved this protocol.

2.1 Sampling and preanalytical phase of testing

A total of 5 mL of blood was collected in tubes containing heparin from each patient. The preanalytical phase was conducted as follows: patient information was enrolled in the CHU informatics system, samples were labeled and centrifuged at 4,000 rpm for 10 min, and from each aliquot, 200 µL of plasma was recovered after centrifugation, and the aliquots were stored in a freezer at −20°C until usage.

2.2 Detection of autoantibodies using immunoblot technique (Blot-17)

At the first stage, ANA was detected by the indirect immunofluorescence assay in HEp-2 cells. This assay helps us to determine the appearance of ANA and orients it toward the antigenic targets. The next step was the identification of specific ANA with immunoblot.

2.2.1 Different components in the kit

ImmunoBlot Kit (AESKUBLOTS® ANA-17 Pro) was used to determine the type of detected ANA in HEp-2000 cells. The kit was purchased from GIGALAB Diagnostic and contained 24 numbered strips. Each strip consisted of a membrane on which different lines of highly purified autoantigens were placed.

2.2.2 Automated analyzer

The AESKUBLOTS® ANA-17 Protest was carried out using an Automated analyzer (HELMED-Automated Processor for ELISA, BLOT and IFA) (Figure 1).

2.2.3 Reading the results

The results were read using AESKU SCAN. This software was connected to a scanner that produces a sheet on which the strips are fixed. After reading the strips, the results were obtained in tables and peaks with positive and negative tests.

2.3 Detection of autoantibodies using immunodot technique (ANA-25 Screen IgG)

After identifying the autoantibody specificities using the immunoblot technique, the samples were then analyzed again using immunodot technique to compare the performance of the two techniques in identifying autoantibody specificities.

2.3.1 Automated analyzer

BlueDiver is an innovative instrument that allows the automation of different stages of the immunodot

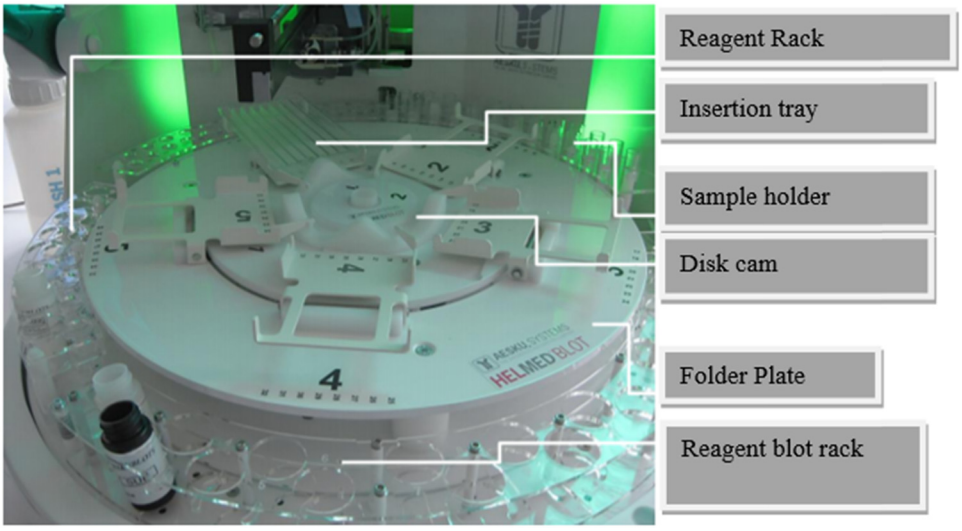

Figure 1: Immunoblot image (Blot-17, HELMED).

technique (Figure 2). The BlueDiver is characterized by reliability, speed, and low cost. Currently, it is used for the diagnosis of autoimmune diseases. The large combination of antigens offers a wide array that allows for the diagnosis of several autoimmune diseases such as conjunctivitis, gastritis, celiac disease, paraneoplastic syndromes, autoimmune hepatitis, primary biliary cholangitis, and vasculitis.

Dr-DOT is a multilingual software that allows for easy interpretation and semiquantitative or quantitative readings of BlueDot kit strips. This software requires only a computer and a BlueScan scanner. Dr-DOT software also contains a database that records the results (numerical values and scanned images).

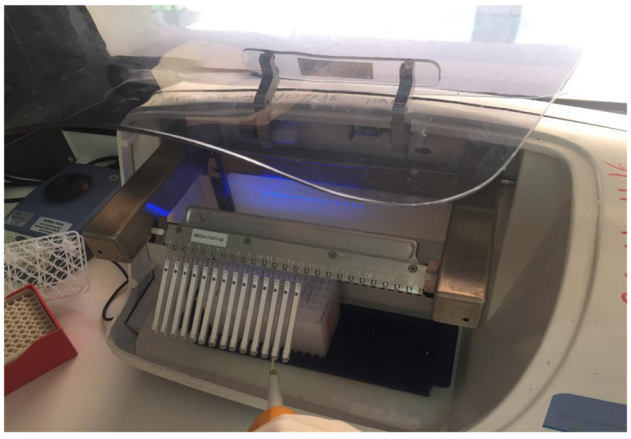

Figure 2: Immunodot image (BlueDiver).

2.4 Statistical analysis

Data were statistically analyzed using the *t*-test. A *p* value less than 0.05 was considered as statistically significant.

3 Results and discussion

3.1 Distribution of patients based on sex

The current study included patients with autoimmune disease including 75% females and 25% males.

This analytical and descriptive research study was conducted in 300 patients of whom 62 were positive for ANA according to the indirect immunofluorescence screening. The results showed a female predominance for positively testing for ANA with 75.81% (47 cases). These results were in agreement with the earlier data [6], where it was reported that the systemic autoimmune diseases exceedingly affected women of the reproductive age. The women's predominance of antinuclear antibodies is explained by the influence of hormonal factors on the immune system. Indeed, estrogen stimulates the humoral immune response, while progesterone and androgens exert a suppressive effect on the humoral and cellular immune response. A high level of 17-beta estradiol was recorded in patients with rheumatoid arthritis and systemic lupus erythematosus [7].

3.2 Distribution of patients according to age

In this study, the age of the patients ranged from 1 to 71 years, with an average age of 41.92 years. Patients who aged between 31 and 46 years were the most affected with a frequency of 0.24 (24%) (Figure 3).

By analyzing the age categories of the studied patients, it was found that the age group between 31 and 46 years was the group most affected by ANA (24%). The study results were in agreement with those reported in early research data showing that systemic diseases frequently affects individuals with an average age of 42 years [8]. Systemic scleroderma (ScS) frequently affects individuals aged between 40 and 50 years [6].

3.3 Distribution of patients according to the type of disease

In the study population, four types of autoimmune diseases were detected. Lupus and Gougerot-Sjögren syndrome were the most frequent autoimmune diseases with frequencies of 40 and 23%, respectively (Figure 4).

In this study, lupus was found to be the most common disease with 40%, followed by Sjögren's syndrome with 23%. The same results were reported in the previous literature, where it was shown that systemic lupus largely affects women during genital activity. An early study reported that patients older than 65 years were subject to ANA diagnosis. In this study, nobody was detected with the currently investigated diseases as reported in the previously published data [9]. According to the most recent data of pediatric rheumatology, juvenile idiopathic arthritis was 5–10 times less common than lupus in people aged 18 years or younger. In the case of the frequency of dermatomyositis in children, the annual incidence has been rarely established. It was estimated to be 0.37/1,00,000 in Finland and 6/1,00,000 in New York before the age of 15 years. According to the reported data, the incidence is around 10 to 20/1,00,000 and the prevalence ranged from 10 to 20/10,000 before the age of 18 years [3].

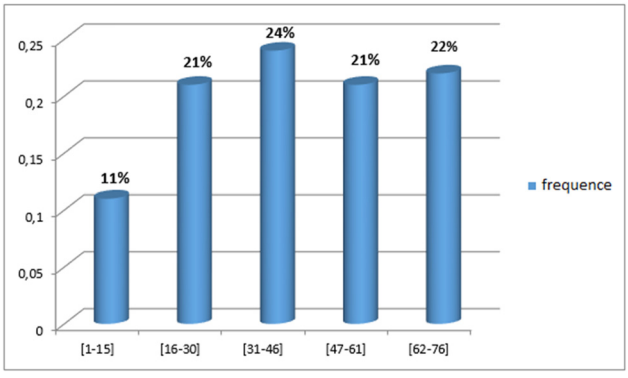

Figure 3: Distribution of patients according to age.

3.4 Immunological profile

All patients were tested positive for ANA according to indirect immunofluorescence screenings. Forty-eight percent (n = 29) of patients showed a speckled immunofluorescence (speckled aspect) and 27% (n = 16) showed a homogeneous immunofluorescence (homogeneous aspect) (Figure 5).

Regarding the immunological profile, an important diversity of immunofluorescence aspects was recorded with a predominance of speckled appearance with 48%, followed by the homogeneous aspect with 27%. Immunofluorescence aspects results point to the antibody specificity before the identification process [10].

Antinuclear antibodies are directed against nuclear constituents, anti-DNA, antinucleosome, antihistone, anti-SSA, and anti-SSB. Antinuclear antibodies were directed against polypeptides fixed in small RNA called YRNA, whether cytoplasmic (70%) or nuclear (30%). These ANAs are markers of lupus, systemic Sjögren syndrome, and undifferentiated connective tissue disease [11]. The reported findings in this study showed that anti-SSA was the most frequent in patients with lupus followed by anti-ds-DNA and anti-histone.

Gougerot-Sjögren syndrome was present in 23% of the studied population. Patients with anti-SSA were the most common (28%), followed by patients with anti-SSB (7%) and anti-histone. The current reports were in accordance with the previously published data showing that patients with anti-SSA antibodies represented 28% of the total affected cases. A total of 13.7% of patients were reported with anti-SSB antibodies. Conversely, anti-SSB and SSA antibodies were present in 94.5 and 54.5% cases, respectively [12].

A total of 76.9% of patients with Sjögren syndrome were positive for anti-SSA antibodies [13]. Anti-SSA and anti-SSB antibodies were identified in 7 and 9% of patients with Sjögren syndrome, respectively [14]. The results of this study reported that the anti-Scl-70 due to scleroderma positive tests were highly abundant with an index of 4.7. These findings were in agreement with the

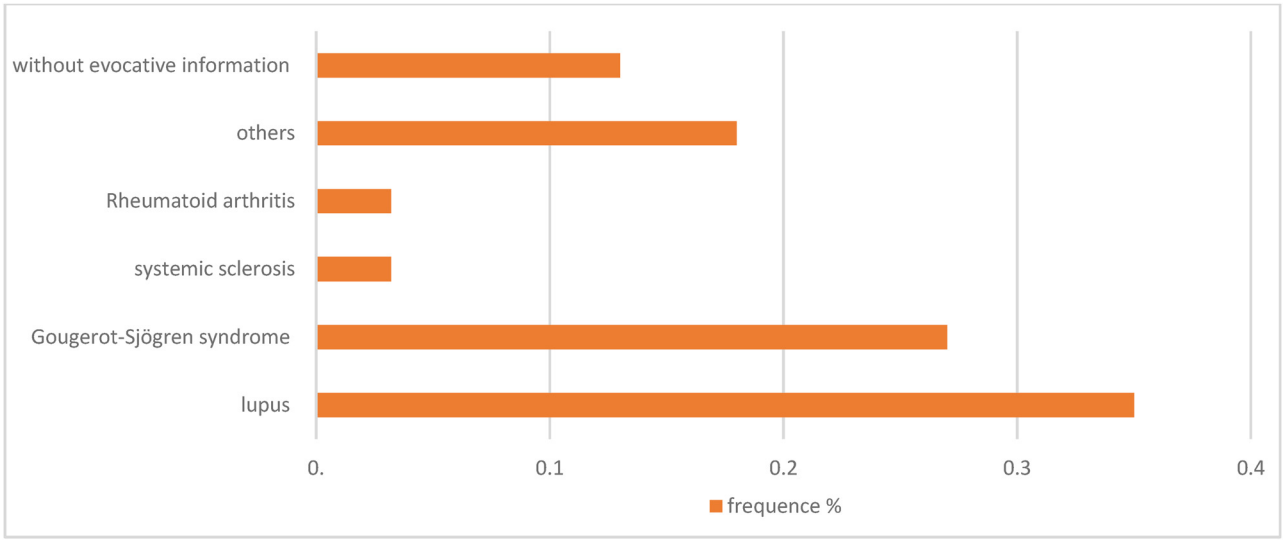

Figure 4: Distribution of patients according to the type of disease.

previously published results [15], in which it was reported that the frequency of anti-Scl-70 Ab was significantly abundant in patients younger than 50 years. Moreover, the results obtained in this study agree with those reported in the previous study, which showed that rheumatoid arthritis and autoimmune thyroiditis, which are severe autoimmune diseases, with serious antinuclear antibodies affect more than 5% of the population in developed countries [16,17].

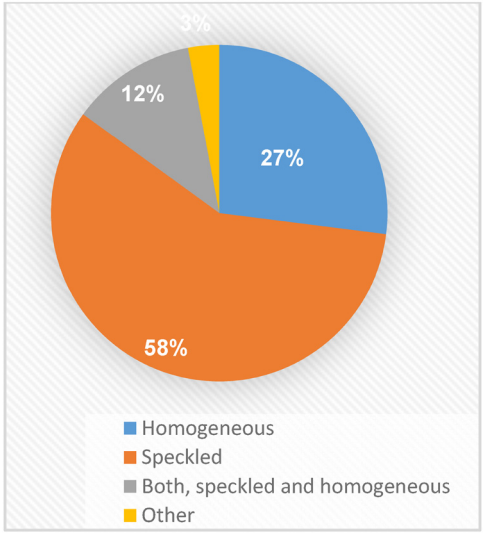

Figure 5: Distribution of antinuclear antibodies aspects.

3.5 Distribution of antinuclear antibodies according to systemic diseases

Anti-SSA antibodies were abundantly present in lupus patients, including nine cases with specific anti-SSA/60, six cases with anti-SSA/Ro 52 and anti-SSB, followed by five cases with anti-DNA and four cases with anti SmD1. In case of patients with Gougerot-Sjögren syndrome, two cases were registered with anti-SSA/60 and SSA/52 followed by two cases with anti-histones and one case with anti-SSB. In case of patients with systemic sclerosis, the only target in the present screening was one case with anti-Scl-70 (Figure 6).

3.6 Study of concordance between immunodot and the immunoblot results

No significant difference between immunodot and immunoblot sensitivity regarding eight antigenic targets including nine cases of SSA/Ro 52, five cases of SSB, two cases of CENPB, and a single case of Jo-1 and Mi-2 each (Table 1).

A significant difference between immunodot and immunoblot sensitivity toward the identification of autoantibodies was recorded for nine antigenic targets ($p < 0.05$): anti-ds-DNA with 25% (eight positive cases were detected by immunodot and six positive cases were detected by immunoblot), antinucleosomes with 46% (13 cases positive were detected by immunodot and seven were detected by immunoblot), anti-histones with 55%

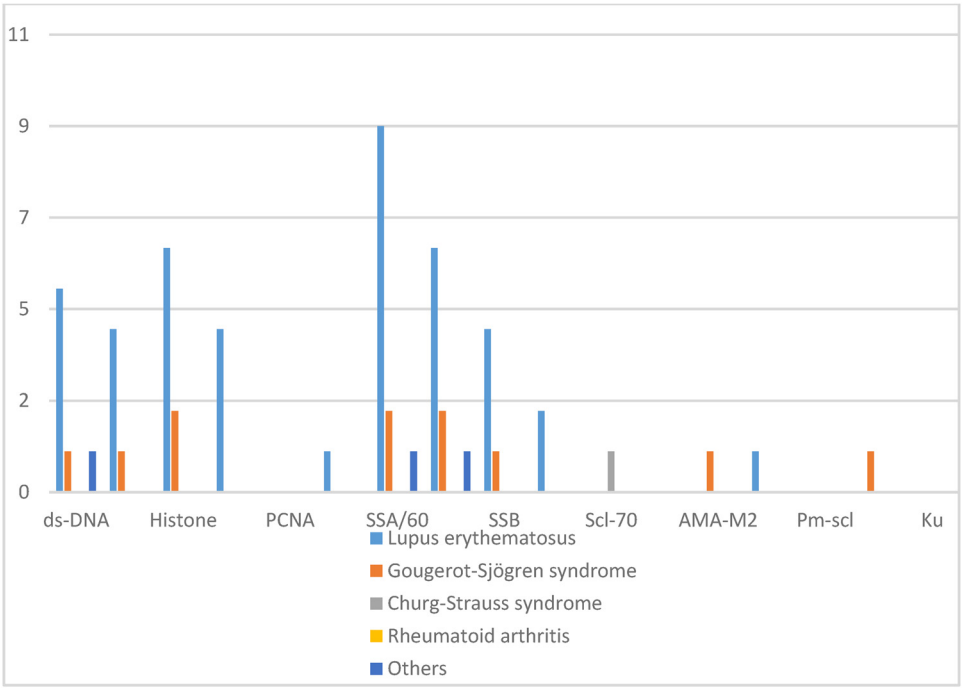

Figure 6: Immunological results according to the presence of ANA in systemic diseases.

(five positive cases were detected by immunodot and nine positive cases were detected by immunoblot), anti-SmD1 with 20% (five positive cases were detected by immunodot and four cases were detected by immunoblot), anti-Rib-Po with 50% (two positive cases were detected by immunodot and one was detected by immunoblot), antis-Scl-70 with 50% (two positive cases were detected by immunodot and one was detected by immunoblot), AMA-M2 with 67% (three positive cases were detected by immunodot and one was detected by immunoblot), and anti-Pm-scl, with one case, was detected by immunodot.

This study aimed to compare Dot-25 and Blot-17. A significant difference was recorded between the performances of the two techniques. The results showed that

Table 1: Study of concordance and discordance between immunodot and immunoblot

	Immunodot		Immunoblot		Concordance dot/blot (%)	Discordance dot/blot (%)
	Number of cases	Percentage	Number of cases	Percentage		
ds-DNA	8	13.00	6	9.60	75.00	25.00
Nucleosome	13	21	7	11	54	46
Histone	5	8	9	15	45	55
SmD1	5	8	4	6	80	20
PCNA	0	0	0	0	100	0
Rib-Po	2	3	1	2	50	50
SSA/60	13	21	11	18	85	15
SSA/Ro 52	9	15	9	15	100	0
SSB	5	8	5	8	100	0
CENP-B	2	3	2	3	100	0
Scl-70	2	3	1	2	50	50
U1-snRP	0	0	0	0	100	0
AMA-M2	3	5	1	2	33	67
Jo-1	1	2	1	2	100	0
Pm-scl	1	2	0	0	0	100
Mi-2	1	2	1	2	100	0
Ku	0		0	0	100	0

the Blot-17 technique was more sensitive regarding certain investigated antinuclear antibodies including antihistones. Conversely, Dot-25 technique was more sensitive versus other antinuclear antibodies (anti-SSA, anti-ds-DNA, and so on).

To the best of our knowledge, no previous study compared efficiency and performance of Dot-25 and Blot-17 techniques versus the same autoantibodies identification. Hence, this study seems to be the first study that highlighted the difference in the sensitivity of these techniques toward the same antinuclear antibodies.

The sensitivity and specificity of different tests used for the detection of autoantibodies can vary from one study to another according to several reasons: First, there is no international standardization of the methods used in the detection; thus, the normal values may vary according to the population study. Therefore, the diagnosis should not be established solely with a single method. The obtained results should always be interpreted taking into account the clinical examination conditions, the patient's history, as well as the results obtained through other methods. No single technique can rule out the possibility of false-positive or false-negative results. Analysis with an indirect immunofluorescence test should be done to confirm the obtained findings.

Both Dot-25 and Blot-17 were equally sensitive against antinuclear antibodies (PCNA, SSA/52, SSB, CENP-B, Jo-1, Mi-2, and so on). There were no significant differences shown between their performances regarding the detection of the antinuclear antibodies. However, a significant difference was reported between the two techniques regarding other antinuclear antibodies including anti-nucleosome, anti-ds-DNA, anti-SSA/52, anti-SSB antihistone, anti-SmD1, anti-SSA, and anti-ds-DNA ($p > 0.05$)).

4 Conclusion

This study was prospectively carried out to assess the sensitivity of immunoblot and immunodot versus the same antinuclear antibodies present in the serum of patients affected by systemic autoimmune diseases. Although both techniques, immunoblot and immunodot, are currently being used to identify the same variety of antinuclear antibodies, they do not have similar performance (sensitivity) versus the same antinuclear antibodies. Hence, it is very important to pay more attention to the performance of techniques used for the identification of antinuclear antibody.

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Data availability statement: Data used to support the findings are incorporated in the manuscript.

References

[1] Chevailler A, Beauvillain C, Carrère F. Dépistage des anticorps dirigéscontre les an tigènes nucléaires solubles. Revue Francophone des Laboratoires. 2006;2006(384):59–70.
[2] Subra J-F. Silice et auto-immunité. Revue Française des Laboratoires. 2004;2004(361):23–5.
[3] Contin-Bordes C, Lazaro E, Pellegrin JL, Viallard JF, Moreau JF, Blanco P. Systemic lupus erythematosus: from pathophysiology to treatment. La Revue de Médecine Interne. 2009;30(12 Suppl):H9–13.
[4] Tan EM. Antinuclear antibodies: diagnostic markers for autoimmune diseases and probes for cell biology. Advances in immunology. Vol. 44, California, USA: Elsevier; 1989. p. 93–151.
[5] Van Venrooij WJ, Maini RN. Manual of biological markers of disease. Boston, USA: Springer Science & Business Media; 2012.
[6] Hachulla E, Launay D. Complications maternelles graves des maladies systémiques auto-immunes. Réanimation. 2007;16(5):393–402.
[7] Cutolo M, Capellino S, Sulli A, Serioli B, Secchi ME, Villaggio B, et al. Estrogens and autoimmune diseases. Ann N Y Acad Sci. 2006;1089(1):538–47.
[8] Mahfoudh A, Khaled A, Chtourou O, Kharfi M, Zeglaoui F, Fazaa B, et al. Chronic lupus erythematosus: 104 Tunisian cases. La Tunisie Medicale. 2010;88(10):742–5.
[9] Pu SJ, Luo SF, Wu YJJ, Cheng HS, Ho HH. The clinical features and prognosis of lupus with disease onset at age 65 and older. Lupus. 2000;9(2):96–100.
[10] André C, Bossuyt X. Systèmes automatisés de lecture des images de fluorescence. Revue Francophone des Laboratoires. 2012;444(2012):13–5.

[11] Meyer O. Critères de classification: mode d'emploi pour le diagnostic de lupus systémique. Revue du Rhumatisme. 2005;72(2):142–9.
[12] Chen N, Zhou M, Dong X, Qu J, Gong F, Han Y, et al. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. Lancet. 2020;395(10223):507–13.
[13] Horvath IF, Szanto A, Papp G, Zeher M. Clinical course, prognosis, and cause of death in primary Sjögren's syndrome. J Immunol Res. 2014;2014:647507.
[14] Friedman JA, Miller EB, Green L, Huszar M, Schattner A. A community-based cohort of 201 consecutive patients with primary Sjögren's syndrome in Israel: Ashkenazi patients compared with those of Sephardic descent. Clin Exp Rheumatol. 2006;23:274–80.
[15] Sato S, Hamaguchi Y, Hasegawa M, Takehara K. Clinical significance of anti-topoisomerase I antibody levels determined by ELISA in systemic sclerosis. Rheumatology. 2001;40(10):1135–40.
[16] Sinha AA, Lopez MT, McDevitt HO. Autoimmune diseases: the failure of self tolerance. Science. 1990;248(4961):1380–8.
[17] Jacobson DL, Gange SJ, Rose NR, Graham NM. Epidemiology and estimated population burden of selected autoimmune diseases in the United States. Clin Immunol Immunopathol. 1997;84(3):223–43.