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Evaluation of the ELISA technique compared to Immunodot in the screening of extractable nuclear antigens antibodies Sm, SSA and SSB

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ABSTRACT: Autoimmune diseases are marked by the presence of more or less specific autoantibodies for each of them. The detection and identification of these autoantibodies seem to be the pillars of the diagnosis. The aim of our study is to evaluate the ELISA technique compared to the immunodot, for the detection of extractable nuclear antigens antibodies Sm, SSA and SSB. This is a retrospective, comparative study of biological diagnostic techniques carried out over 6 months. 86 sera from patients tested by the immunodot for the detection of extractable nuclear antigens antibodies were analyzed by an ELISA test (AESKULISA) for the detection of anti-Sm, anti-SSA and anti-SSB antibodies. Sensitivities, specificities, PPVs, NPVs and the correlation index were calculated respectively for each of the three kits AESKULISA Sm (57.14%, 97.46%, 66.6%, 97.46%, 94%), AESKULISA SSA (53.84 %, 97.87%, 95.45%, 71.87%, 77.90%) and AESKULISA SSB (16.66%, 100%, 100%, 81.92%, 82.55%). Our study found low levels of sensitivity of the ELISA technique compared to the immunodot, which could be explained by the nature of the antigenic substrates, the coating procedures and the cut-off levels used by kits manufacturers. The immunodot appears to be more sensitive and more specific for the detection of anti-SSB antibodies and more sensitive for the detection of anti-SSA 52kDa antibodies. Indeed, a combination of two or more methods is to be recommended in order to optimize the relevance of the diagnostic test for the screening of anti-ENA antibodies.

KEYWORDS: autoantibodies, autoantigens, antinuclear, horseradish peroxidase, fluorescence.

1. INTRODUCTION

Autoimmune diseases (AIDs) affect around 5% of the population and represent a family of at least 80 diseases which share a common pathogenesis, which is an immune attack against organs of the body. Many diseases are increasing in frequency in industrialized countries. These complex diseases result from the interplay between genetics, epigenetics and environmental factors. Infections are risk factors for a wide range of autoimmune diseases. Treatment of AID improved dramatically during the second half of the twentieth century, but was hampered because the disease often progresses before a clinical diagnosis is possible. The presence of specific autoantibodies is a fundamental parameter for the diagnosis of AID and is part of the classification criteria for many systemic autoimmune diseases. Some good examples are the detection of anti-nuclear (ANA), anti-Sm, anti-dsDNA and anticardiolipin (aCL) antibodies in systemic lupus erythematosus (SLE), the presence of anti-SSA and anti-SSA autoantibodies. SSB in Sjögren's syndrome and the determination of aCL and anti β 2-glycoprotein in antiphospholipid syndrome. Some autoantibodies can be detected several years before the onset of illness, so they act as markers for future illness in currently healthy individuals. A number of techniques for testing autoantibodies have been developed over time. Indirect immunofluorescence (IFA) is conventionally used for the detection of antinuclear antibodies (ANA) but does not allow precise identification of these autoantibodies, hence the interest of immunoenzymatic techniques, in particular ELISA techniques and Immunodot [1], [2], [3], [4], [5], [6], [7].

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The aim of our study is to evaluate the ELISA technique (AESKULISA) compared to the immunodot (ANA 12 Line Dot), in order to optimize the choice of the method to be used for the identification of the anti-soluble nuclear antigen antibodies. Sm, SSA, SSB, in systemic lupus erythematosus (SLE) and in Sjogren's syndrome (SS).

2. MATERIAL AND METHODS

This is a comparative study of biological diagnostic techniques, spread over a period of 6 months. The aim of our study is to compare the characteristics of the AESKULISA technique (ELISA) compared to the ANA 12 Line Dot technique (Immunodot), for the screening of anti-Sm, anti-SSA and anti-SSB autoantibodies. We studied 86 serum samples stored at -20°C, provided by the serum library of the Immuno-Serology Laboratory of the Ibn Rochd University Hospital in Casablanca, of which 43 sera were positive for one or more of the following 3 auto-antibodies (anti-Sm, anti-SSA, anti-SSB), and 43 negative sera, all tested by the ANA 12 Line Dot technique (Immunodot).

The sera used in our study come from consultant or hospitalized patients in the Dermatology, Internal Medicine, or Rheumatology departments of the Ibn Rochd University Hospital in Casablanca, 90% of which are women.

2.1. IMMUNODOT- ANA 12 LINE DOT

ANA 12 Line Dot is a sensitive Immunodot assay for the determination of anti-DNA, nucleosomes, Sm, ribosomes, histones, RNP, SSA 60kDa, SSA 52kDa, SSB, Scl-70, CENP-B and Jo-1 in human serum or plasma.

ANA 12 Line Dot includes 20 numbered test strips, containing 12 line dots coated with specific antigens: dsDNA, nucleosomes, Sm, P0, histone complex, RNP (A, C, 68kDa), SSA 60kDa, SSA 52kDa, SSB, Scl-70, CENP-B and Jo-1, respectively. Three test lines serve as Function Control, Negative Control and Cut-off.

Patient sera and bands are incubated in the test tray. During the first incubation, antibodies from the patient sample bind to target autoantigens immobilized on the solid phase of the bands. After an incubation period of 45 minutes at room temperature with stirring, the unbound serum components are removed by a washing step.

Bound antibodies react specifically with anti-human IgG conjugated to horseradish peroxidase. After an incubation period of 45 minutes at room temperature with stirring, the excess conjugate is separated from the solid phase immune complexes by an additional washing step.

Horseradish peroxidase converts the added solution of the added tetra-methyl-benzidine (TMB) substrate, which is colorless, to a dark blue precipitating product. After 10 to 12 minutes with stirring, the reaction is stopped by a washing step.

The strips are dried for at least 30 min by pressing the reagent side onto absorbent paper. Different patterns of lines become visible by the antibodies present in the serum samples of the patients. The bands are interpreted using the pattern template provided by the kit.

Results considered positive are those for which the color of the test point is more intense than the color of the cut-off point.

2.2. ELISA - AESKULISA KIT

The serum samples diluted to 1: 101 are incubated in the microplates sensitized with the specific antigen (Sm or SSA or SSB).

Antibodies in the patient sample bind to the antigen on the microplate. The unbound fraction is then removed by a washing step, anti-human IgG immunoglobulins labeled with horseradish peroxidase (conjugate) react after incubation with the antigen-antibody complex fixed on the microplates. The unbound conjugate is then washed away. The addition of TMB substrate (tetra-methyl-benzidine) induces a colored enzymatic reaction (blue), stopped by the addition of hydrochloric acid (the color then turns yellow).

The intensity of the color that develops from the chromogen depends on the amount of the conjugate bound to the antigen-antibody complex and is proportional to the initial concentration of each antibody in the sample tested.

Table 1. The characteristics of the two techniques AESKULISA and ANA 12 Line Dot tested in our study

CHARACTERISTICS	AESKULISA (ELISA)	ANA 12 Line Dot (Immunodot)
Technical	Manual	Manual
Principle	Immunoenzymatic on sensitized microplates	Immunodot on sensitized paper strips
Methods	Qualitative Semi-quantitative	Qualitative
Composition of the plate Or strips	Microplates with wells sensitized by the antigen Ex: Sm, SSA, SSB	Strips sensitized by 12 soluble antigens (DsDNA, nucleosomes, Sm, P0, histone complex, RNP (A, C,68 kDa), SSA 60kDa, SSA 52kDa, SSB, Scl-70, CENP-B and Jo-1)
Conjugate	Anti-human IgG conjugated to horseradish peroxidase	Anti-human IgG conjugated to horseradish peroxidase
Sample	Human serum	Human serum
Sample volume	10µl of serum diluted to 1: 101	15µl of pure serum per strip
Validation procedure of the technique	Positive, negative controls And Cut-off	Clearly visible (+) control, faintly visible cut-off, less intense (-) control than cut-off
Results interpretation	Threshold index = OD sample/OD serum threshold Or reference curve	Positive result if the color intensity of the antigen bands is greater than or equal to the Cut-off band
Duration of analysis	95 minutes	2 hours 30 minutes
Number of tests	96 / plate	20 / kit
Sensitivity (%)	Not mentioned	89%
Specificity (%)	Not mentioned	96%

We calculated the characteristics (correlation, sensitivity, specificity, PPV, NPV, and Youden's index) of the AESKULISA assay, compared to the performance of the ANA 12 LINE DOT assay.

The sensibility:

Is the probability that the test will be positive if autoantibodies are present in the patient's serum: Sensitivity = $\frac{VP}{VP+FN}$

The specificity:

Is the probability of obtaining a negative test in patients who do not have autoantibodies in their sera: Specificity = $\frac{VN}{VN+FP}$

Positive predictive value (PPV):

Is the probability that autoantibodies are present in the serum when the test is positive: PPV = $\frac{VP}{VP+FP}$

The negative predictive value (NPV):

Is the probability that autoantibodies are not present in the serum when the test is negative: NPV =$\frac{VN}{VN+FN}$

YOUDEN index (YI):

Measures the efficiency of the test, **YI = sensitivity + specificity - 1**

Negative index = ineffective test

Index approaches 1 = effective test

Correlation between the 2 techniques:

$$\frac{VP+VN}{T}$$

3. RESULTS

3.1. RESULTS OBTAINED BY THE ANA 12 LINE DOT TECHNIQUE

A set of 86 sera were tested to evaluate the ELISA technique (AESKULISA) compared to the reference technique Immunodot (ANA 12 Line Dot).

Forty-three sera were found to be positive in one or more antibodies (7 anti-Sm, 39 anti-SSA, 18 anti-SSB the total of positive sera is greater than 43, this is explained by the fact that some sera are positive for 2 or even 3 different autoantibodies) and 43 negatives by the ANA 12 Line Dot method.

3.2. RESULTS OBTAINED BY THE AESKULISA KIT

We tested the same sera with the AESKULISA (ELISA) technique and we obtained the following results:

AESKULISA Sm 6 positive and 80 negative

AESKULISA SSA 22 positive and 64 negative

AESKULISA SSB 3 positive and 83 negative

Table 2. Results obtained by both techniques for the anti-Sm antibodies

		ANA 12 LINE DOT			Total
		Positive	Equivocal	Negative	
AESKULISA® Sm	Positive	4	0	1	5
	Equivocal	0	0	1	1
	Negative	2	0	78	80
Total		6	0	80	86

For the anti-Sm antibodies, we obtained a single equivocal serum by the AESKULISA technique, which we counted positive according to the manufacturer's recommendations.

Table 3. Results obtained after counting the equivocal serum

		ANA 12 LINE DOT		Total
		Positive	Negative	
AESKULISA® Sm	Positive	4	2	6
	Negative	3	77	80
Total		7	79	86

From the results already cited, we calculated the characteristics of the AESKULISA Sm test (ELISA) compared to the ANA 12 LINE DOT test.

Thus, we obtained a sensitivity of **57.14%**, a specificity of **97.46%**, positive predictive values and negative predictive value respectively of 66.6% and 96.25%, a Youden index of 0.54 and a correlation of 94%.

Table 4. Characteristics of the AESKULISA® Sm assay compared to the ANA 12 Line Dot assay for anti-Sm antibodies

Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Youden Index	Correlation (%)
57.14	97.46	66.6	96.25	0.55	94.19

***PPV**: Positive predictive value **NPV**: Negative predictive value*

Table 5. Results obtained by both techniques for anti-SSA antibodies

		ANA 12 LINE DOT			Total
		Positive	Equivocal	Negative	
AESKULISA® SSA	Positive	18	0	1	19
	Equivocal	3	0	0	3
	Negative	18	0	46	64
Total		39	0	47	86

For the anti-SSA antibodies, we obtained 3 equivocal sera by the AESKULISA test which we counted positive according to the manufacturer's recommendations.

Table 6. Results after counting equivocal sera

		ANA 12 LINE DOT		Total
		Positive	Negative	
AESKULISA® SSA	Positive	21	1	22
	Negative	18	46	64
Total		39	47	86

From the results already cited, we calculated the characteristics of the AESKULISA SSA (ELISA) test compared to the ANA 12 LINE DOT test.

Thus, we found a sensitivity of **53.84%**, a specificity of **97.87%**, positive predictive values and negative predictive values of 95.45% and 71.87% respectively, a Youden index of 0.51 and a correlation of 77.90%.

Table 7. Characteristics of the AESKULISA® SSA test compared to the ANA 12 Line Dot for anti-SSA antibodies

Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Youden Index	Correlation (%)
53.84	97.87	95.45	71.87	0.51	77.90

PPV: *Positive predictive value* ***NPV****: Negative predictive value*

Table 8. Results obtained by both techniques for anti-SSB antibodies

		ANA 12 LINE DOT		Total
		Positive	Negative	
AESKULISA® SSB	Positive	3	0	3
	Negative	15	68	83
Total		18	68	86

From the results already cited, we calculated the characteristics of the AESKULISA SSB (ELISA) test compared to the ANA 12 LINE DOT test.

Thus we found a sensitivity of **16.66%**, a specificity of **100%**, positive predictive values and negative predictive values of 100% and 81.92% respectively, a Youden index of 0.16 and a correlation of 82.55%.

Table 9. Characteristics of the AESKULISA® SSB test compared to the ANA 12 Line Dot test for anti-SSB antibodies

Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Youden Index	Correlation (%)
16.66	100	100	81.92	0.16	82.55

PPV: *Positive predictive value* ***NPV:*** *Negative predictive value*

4. DISCUSSION

Anti-ENA antibodies are very useful markers for the diagnosis of various autoimmune diseases. Their screening is generally carried out by indirect immunofluorescence (IFA), using HEp-2 cell lines as a substrate. However, the accuracy of this method is limited by the inter-laboratory variability of the results, due to the performance of the procedure as well as the subjectivity of the interpretation, hence the development of immunoenzymatic techniques [10].

The identification of anti-ENA can be carried out by different methods variously used by laboratories. The oldest techniques use agar immunodiffusion (Ouchterlony technique) using human or animal antigens. The sensitivity of this test is average but its specificity is good and it remains the reference method, even if it is not very standardized, hence the advent of immunoenzymatic techniques (ELISA or Immunodot) which use natural animal antigens or human antigens that could be natural or recombinant, and which perform better with varying sensitivities and specificities depending on the quality of the antigen used. These are the techniques most used in practice by laboratories [13], [12], [9].

In this work, we carried out a comparative study between two immunoenzymatic techniques, ELISA-AESKULISA and Immunodot-ANA 12 LINE DOT for the screening of anti-ENA antibodies Sm, SSA and SSB, on a set of 86 sera provided by the Immuno-Serology Laboratory of Ibn Rochd University Hospital. These sera were sent to the Immuno-Serology laboratory of Ibn Rochd University Hospital for the detection of anti-ENA antibodies by immunodot.

The results obtained made it possible to calculate the sensitivity and specificity as well as the PPV and NPV of the "AESKULISA" kit, compared with the "ANA 12 Line Dot" kit.

By referring to the data provided by the manufacturer, the characteristics of the ANA 12 Line Dot kit give a sensitivity of 89% and a specificity of 96% for the screening of anti-ENA antibodies.

In our study, the sensitivities and specificities obtained by the AESKULISA-Sm, AESKULISA-SSA and AESKULISA-SSB kits were respectively (57.14%, 53%, and 16.66%) and (97.74%, 97.87%, 100%).

The low levels of sensitivity found by the AESKULISA tests for the 3 antibodies can be explained by possibly higher cut-off levels, chosen by the manufacturers of the AESKULISA kits, in order to maintain an acceptable level of diagnostic specificity. This can also justify the 3 equivocal sera which we obtained by the AESKULISA SSA kit and which were positive by the immunodot (ANA 12 Line Dot).

The results of our study join those obtained by a comparative study carried out by Manoussakis et al. [12], of five methods, including ELISA and immunodot, used to detect anti-SSA antibodies in 93 sera from consultant or hospitalized patients. The ELISA for anti-SSA antibodies showed comparable specificity (95%) to ours (98%), and slightly higher sensitivity (72%), compared to ours (53%).

On the other hand, regarding the screening of anti-SSB antibodies, this study revealed that the immunoblotting of the recombinant SSB protein or whole cell extract is the most sensitive and specific for the detection of anti-SSB antibodies [11].

Another study was carried out of 76 sera, this time to evaluate the characteristics of the immunodot technique for the identification of anti-ENA antibodies, including anti-SSA and anti-SSB antibodies, in patients with connective tissue disease. The sera were tested by IFA, immunodot and by ELISA (sensitized with purified SSA and SSB antigens). Of the 76 sera tested, 35 were positive by immunodot and 33 by ELISA. The sensitivity, specificity, PPV and NPV of the immunodot test were calculated by comparison with the ELISA technique, considered as the reference technique [8].

Table 10. Results of the comparative study of Immunodot versus ELISA

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Anti-SSA	100	98.6	80	100
Anti-SSB	100	100	100	100

Furthermore, the low level of sensitivity observed in our study may be due to the nature of the antigen used in our study, which is recombinant SSA-52kDa and SSB antigens. Since anti-SSA and anti-SSB recognize polypeptides attached to small RNAs called YRNAs; conformational epitopes, the purified antigens thus present an advantage for screening anti-SSA and anti-SSB [9], [16], [17].

Another study was performed on 1513 patients of which 315 were diagnosed with autoimmune disease and 1198 healthy subjects, to assess the analytical precision of five enzyme immunoassay (EIA) techniques, which use different antigenic substrates and different coating procedures, for screening ANA. The results were compared with those obtained with the method of IFA reference. They demonstrate that the commercially available EIA kits show different levels of sensitivity and specificity. Some kits have comparable and even better diagnostic accuracy than the IFA method. Therefore, they could be used as an alternative screening method to IFA. However, others do not guarantee acceptable results. This comes down to the fact that these tests use different methods to prepare the antigenic substrate, so they exhibit different levels of sensitivity and specificity [11], [14].

It is widely known that certain autoantibodies directed against highly soluble or poorly represented antigens on cell substrates, such as SSA and Jo-1, can be detected more easily by solid phase assays [10].

Table 11. Nature of the antigens used in the AESKULISA and ANA 12 Line Dot techniques

AESKULISA SSA 60kDa	AESKULISA SSA 52kDa	AESKULISA SSB	AESKULISA Sm	ANA 12 Line Dot
Native human antigen	Recombinant human antigen	Recombinant human antigen	Native human antigen	Native antigen

Recombinant antigens have a limit of detection which is due to the absence of post-translational modification such as glycosylation, acetylation and phosphorylation [9], [16]. This could partly explain the low sensitivities of the tests used in our study for the detection of anti-SSA and anti-SSB antibodies. Moreover, a comparative study revealed that the ELISA-VARELISA anti-SSA kit is a sensitive test, but not enough to detect anti-SSA 52kDa antibodies (a sensitivity of 100% for SSA 60kDa and 73% for SSA 52kDa). On the other hand, the western blot turns out to be more sensitive than ELISA-VARELISA for the detection of 52kDa anti-SSA. For these reasons, a combination of two or more methods is recommended for the detection of anti-SSA antibodies to be associated with the clinical characteristics of the patient for the evaluation of the relevance of the diagnostic test [14].

The immunodot uses the principle of immobilization of the antigen on a nitrocellulose membrane, capable of retaining a greater number of protein molecules than polystyrene, and has advantages over the mode of antigen binding used in the technique. ELISA which is random and can induce conformational modifications of biomolecules, resulting in loss of epitopes linked to this configuration [9]. This may indeed explain the low sensitivity rates of the ELISA technique compared to the immunodot observed in our study.

The use of a particular antigenic substrate adsorbed to the wells allows better conservation of the conformational epitopes recognized by the antinuclear antibodies. Instead of a complex antigenic substrate (many purified antigens added to the mixture of nuclear antigens) the sensitivity of the kit is generally less when the composition of the kit is multi-antigenic (antigen competition and alteration of conformational epitopes).

In addition, laboratory operators should be aware of the type of antigenic substrate, and the coating procedures used by the manufacturer, bearing in mind that the complexity of the antigenic substrate is not an absolute guarantee of good performance [10], [15].

Moreover, and concerning PPVs and NPVs, our results show PPVs of the SSA and SSB kits higher than those found for the Sm kit.

Our study also revealed a total overall correlation coefficient of 85% between the two techniques AESKULISA and ANA 12 Line Dot.

The Youden index obtained for the three AESKULISA kits ranges from 0.16 to 0.5. It must therefore be improved, in order to support the possible interest of the AESKULISA technique for guiding and putting a diagnosis.

Finally, the differences in sensitivities and specificities observed between the two techniques in our study may be due to the different molecular approaches used by companies to prepare the antigen because, depending on the method used, it is possible to detect autoantibodies directed against different epitopes of the same complex antigen exhibiting a linear or three-dimensional conformation.

In addition, the sera used in our study come from the specimen bank of the Immuno-Serology Laboratory, where they were kept for an average of 2 months. The sera would have undergone probable freezing-thawing to carry out any checks. The use

of fresh sera would have significantly improved the results of our study. Some equivocal results could not be checked because the reagents at our disposal were insufficient.

However, further studies should be performed analyzing other types of anti-ENA antibodies while making use of patient clinical data, as it would be best to refer to the results of the IFA.

The accuracy of a test is critical for early diagnosis. A false positive or false negative test may be responsible for additional costs due to repeating confirmation tests and/or unnecessary additional investigations [10]. Careful evaluation of the various kits on the market is therefore desirable before including any of these methods in the diagnosis routine of connective tissue diseases.

5. CONCLUSION

The diagnosis of connective tissue diseases is based on clinical, radiological data, and laboratory tests. The presence of specific autoantibodies is one of the fundamental parameters.

Indirect immunofluorescence is the technique conventionally used for the detection of ANA, but does not allow precise identification of these autoantibodies. Hence the interest of immunoenzymatic techniques in particular ELISA and Immunodot, which occupy a large place in the current practice of serology laboratories, and testify to a great performance with sensitivity and specificities which vary according to the quality of the antigen used and the coating procedures. Besides the low price and the availability of reagents, ELISA remains a simple, fast technique and gives good results.

Nevertheless, other studies must be carried out by analyzing other types of anti-ENA antibodies, by the ELISA method while exploiting the clinical data of the patients, in order to be able to judge its performance in the detection of these auto-antibodies.

The different molecular approaches used by manufacturers to prepare the antigen is a key parameter, which defines the characteristics of a test. It is therefore desirable to thoroughly evaluate the various kits on the market before including any of these methods in the biological diagnosis.

The combination of two or more methods is to be recommended in association with the clinical data of the patients for an appropriate evaluation of the relevance of the diagnostic test.

CONFLICT OF INTEREST

The authors declare that they haven't known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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