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ORIGINAL ARTICLE

Diagnostic value of Immunoblotting assay for Determination of anti-nuclear antibodies in rheumatic diseases

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ABSTRACT

Antinuclear antibodies (ANAs) are common features of autoimmune connective tissue diseases. There are newer techniques that are continuously put forward to facilitate diagnosis in connective tissue disease (CTD) patients.Immunofluorescence (IF) is the most used and "gold standard" test for diagnosis, and the other is Enzyme-Linked Immunosorbent Assay (ELISA) as a routine test. For Immunoblotting (IB) assay, euroimmune ANA profiles are used, which provide a qualitative in vitro assay for human autoantibodies to 15 different antigens. This study was conducted to compare three techniques (IF, IB, ELISA) in detection of ANA. If the sensitivity and specificity of IB superior to other methods, we can replace IF and ELISA with IB. An analytical cross-sectional study of 85 sera from patients with Systemic lupus erythematous (SLE), Systemic sclerosis (SSc) and Dermatomyosities(DM) was undertaken at rheumatology and nephrology department and clinic of Emam Reza hospital from 2010-11-01 to 2011-10-30. Sera collected and stored at -80˚c. Then they were used to detection of ANA with three techniques. Of all sera, 63 (74.1%) were ELISA positive and 22 (25.9%) were negative ELISA. Seventy four (87.1%) IF and IB positive and 11 (12.6%) IF and IB negative were observed. The sensitivity and specificity of IB in comparison with IF was 98.65% and 90.91%, respectively. In comparison with ELISA, we found 93.65% and 31.82% of sensitivity and specificity for IB method. Immunoblotting has high sensitivity and specificity, and it can be used as a choice test in screening of ANA.

Key words: *Connective tissue diseases, Enzyme-Linked Immunosorbent Assay,Fluorescent Antibody Technique, Immunoblotting*

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INTRODUCTION

Identifying individuals who are at increased risk of autoimmune diseases is important from the aspects of early detection, follow-up and prevention with immunomodulatory therapy. [1] Anti-nuclear antibody (ANA) is found in the serum of patients with connective tissue autoimmune diseases. [2] These antibodies may also have a role in diagnosing non-rheumatologic autoimmune diseases. [3-5]

Different methods are available for the detection of ANA and the modern techniques are also being developed, by which the diagnosis and treatment of connective tissue diseases (CTD) would be easier. [6] The immunofluorescence (IF) method is progressively used to assess ANA and this method is gold standard in diagnosing ANA. [5, 6] Difficulties in interpretation of the results and the lack of a standard

method among different laboratories are outlined as known limitations of IF method. [8,9] However, the IF method is considered as the first-line screening test because of its cheapness, simplicity and high sensitivity and specificity. [5,10]

ELISA is another diagnostic procedure that has multiple varieties. Each methods have own benefits and limitations, so that ELISA may not be detect low titer ANA, [5] however, some studies have been reported that its sensitivity is higher than the IF. [7,8] Determining the specific auto antibodies is from the advantages of ELISA, but this method can detect only one type of auto antibody against specific antigen such as dsDNA or Sm and several different kits should be used to determine various antibodies. [5]

Immunoblotting or Western blotting is a new diagnostic technique in which the ANA autoimmune profile is used and, the antibodies are employed for detection of this disease with the mechanism of antibody-antigen interaction of target protein among a number of other proteins. Afterward, the electrophoresis is applied to separate proteins. [5,6] It is a sensitive test for evaluating ANA which is able to detect 15 types'antibodies and interpreted automatically. [6] The aim of this study was to compare the results of three laboratory methods ELISA, immunoblotting (IB), and immunofluorescence (IF) in the detection of ANA to introduce the IB as an alternative method if its diagnostic value would be acceptable.

MATERIALS AND METHODS

The study is a descriptive cross-sectional study on 90 patients with lupus, scleroderma and inflammatory Myopathies diagnosed by ACR (American College Rheumatology Association) criteria, who were admitted to the rheumatology and nephrology clinic of Imam Reza hospital in Tabriz-Iran from February 2011 until February 2012. The patients were examined for the presence of ANA. Patients were enrolled in the study after obtaining written consent, without age limitation and specific inclusion and exclusion criteria. All steps of the study were performed in accordance with the Declaration of Helsinki and approved by ethics committee of Tabriz University of Medical Sciences. It is noteworthy that patients did not receive any additional intervention in the course of their treatment and there was no cost imposed to the patients.

Sampling

After fasting of patients for 12 hours, 5 ml of blood sample was collected and immediately centrifuged at low speed for separation of the sera. After separation, the serum samples were sent to the laboratory and stored at -80 ˚C until measuring ANA. The assays were performed by the methods of ELISA, immunofluorescence, and immunoblotting in Laboratory of Immunology Research Center, Tabriz University of Medical Sciences.

IF method

All immunofluorescence samples obtained from patients were thawed at room temperature and diluted by phosphate buffer in the ratio of 1 to 100 and then placed on special slides covered with HEp-2 cells. (2,11) Incubation and washing steps of samples were standardized according to the slides manufacturer's instructions. Complexes of antigen-antibody were detected by antibodies against IgA, IgG, and IgM and evaluated by a special microscope. For better results, a single microscope and a certain professional evaluator was used for all subjects. [2] Finally, results of IF were determined as positive and negative findings through nuclear or cytoplasmic fluorescence under microscopic observations (Figure 1). (4)

ELISA method

For ELISA method, serum samples were diluted by the dilution buffer with a ratio of 1 to 100, and steps of samples' preparation and incubation were conducted using the specific ELISA assay kit. Then, the positive and negative cases of this test were determined in the base of cutoff point -0.9 according to the kit manufacturer's instructions.

IB method

In IB assay method, a kit containing cellulose acetate strip coated with 15 antigens of different types was used. In this assay, each lab kit is sufficient for 16 patients. Briefly, serum samples were diluted and incubated in accordance with procedures recommended by the manufacturer of the kit and then the strips were undergone the sufficient washing period. The reaction of patient's serum with antigens-coated strips was visible as stained bands on strips. The strips were dried, and placed on the sticky plate and scanned and then inserted into special interpretation software. The results of the analysis software were obtained as quantitative titer of type of auto-antibody against a specific antigen and interpreted by the software (Figure 2). In this method, there was no cutoff point for the positive results. When the band is appeared in a particular area, the result of test is considered as positive. (6,12) For evaluating the value of immunoblotting and comparing it with the IF as a gold standard method and with ELISA as a conventional method, the sensitivity and specificity of this test were calculated with a 95% confidence interval.

Statistical analysis: Data obtained from this study was analyzed using a statistical software SPSS (ver.20). Results were expressed by means of descriptive statistics (frequency- percentage and mean ± SD). Chi-square and diagnostic value (sensitivity and specificity) tests were used. A P value less than 0.05 was considered as significant in all cases.

RESULTS

The gender and disease information for patients have been shown in figure 1. The mean age of patients was 35.41 ± 9.82 years of age with a minimum age of 18 and maximum of 60 years. After detecting tests for ANA with three different methods (also see figure 2 and figure 3), following results were obtained:

Of 74 cases of SLE patients, 55 patients (74.3 %) were positive and 19 cases (25.7%) were negative by the ELISA method; 65 cases (87.7%) were positive and 9 cases (12.2 %) were negative by the IF Method; and 65 patients (87.7 %) were positive and 9 patients (12.2%) were negative with the IB method.

In addition, Of 9 patients with SSc, 8 cases (88.9%) were positive and one case (11.1%) were negative by the ELISA method; 8 cases (88.9%) were positive and one case (11.1 %) were negative by the IF method; and 8 8 cases (88.9%) were positive and one case (11.1 %) were negative by the IB method.

In dermatomyositis cases, none of them were diagnosed with the method of ELISA; while only one positive and one negative case was diagnosed and reported by the methods of IF and IB. Fourteen cases of patients with negative ELISA and positive IB were suffered from lupus and one case was suffered from dermatomyositis. The results of IF and ELISA methods are shown in table 1.

To determine the diagnostic value of IB, it was compared with conventional ELISA and standard IF:In comparison to ELISA and IF methods, IB sensitivity were 93.65% and 98.65% and its specificity were 31.82% and 90.91%, respectively. In addition, the positive predictive value of the IB, in comparison with ELISA and IF, were 79.73% and 98.65% and its negative predictive value were 63.64% and 90.91%, respectively (table 1).

DISCUSSION

In the present study, we compared the IB method with IF (as a standard screening test) and with ELISA (as a screening routine test). Based on statistical analysis performed, as compared with ELISA, the sensitivity and specificity of immunoblotting method were the 93.65% and 31.82%, respectively and this was due to false negative cases reported with ELISA. On the other hand, in comparison with IF, immunoblottinghad the sensitivity of 98.65% and specificity of 90.91%, respectively. In a study for the screening of ANA in 224 subjects, Copple et al have reported the sensitivity of 80% for IF and the sensitivity of 96% for ELISA method. [8]. In another study, Darnoiseuus and colleagues compared the ELISA and immunoblotting methods, and the results of their study introduced the immunoblotting as an appropriate method for the detection of antibodies against specific antigens. [13] The results of our study are consistent with their results.

Westgeest and colleagues used the IF and IB methods on 200 patients with autoimmune disease in their study. They reported that the sensitivity and specificity for IF method were 54% and 82% and for IB method were 39% and 79%, respectively. In addition, the combination of these two methods increased the specificity to 97%, and decreased the sensitivity to 24%. In this study, both methods of IF and IB were reported as useful methods in discrimination and differentiation of rheumatologic patients. The sensitivity and specificity of IF were higher than those of IB; however there was no consistency between the results of two methods. Therefore, as a screening test, IF may has some preference to IB, but the simultaneous use of both methods is recommended. [4]

Furthermore, the study of Hyashi and his colleagues in Japan for comparison between ELISA and IF, revealed the sensitivity and specificity of 92 % and 65% for IF and 93% and 79% for ELISA, respectively. [14] Since ELISA can be performed with different methods (by application of ELISA specific antigen), it is not surprising that the sensitivity and specificity of this method be calculated in a wide range in various studies; so that the sensitivity range of this method have been reported from 69 to 98% and the specificity from 81 to 98%. [5] The results of IB methods for screening patients with autoimmune diseases and diagnosis of common-used specific antibodies were varied. Some of these studies are mentioned here:

Bridges and colleagues conducted a study in order to compare the results of three tests immunoblotting, immune diffusion and enzyme immunoassay (EIA) for detection of antibodies against nuclear antigens extracted from 500 serum samples. According the results of their study, the sensitivity and specificity for the combination of two methods IB and EIA were 100% and 99%, respectively. [15] In addition, Prince and colleagues have compared the IB and ELISA methods for specific cytoplasmic antigens in 90 patients

with autoimmune connective tissue disease; they have reported the sensitivity of 100% for the IB test to detect specific antibodies. However, this parameter was about 89% for detection of the Scl-70 [16]. In another study, Tamby et al found that the value of IB test was greater than those of IF and ELISA methods in detecting anti-topoisomerase I antibody in patients with SSc. Therefore, the authors' suggestion was the concomitant application of both ELISA and IB as a routine test. [17]

In addition, Dahle and his colleagues studied on 3079 serum samples submitted for screening of ANA by three different tests of IF, ELISA and immunodiffusion. They found that the IF test alone is not sufficient for the screening of auto-antibodies including anti-SSA, and it is also needed to perform other complementary tests such as antigen-specific EIA. The positive predictive value of IF was low in this study and it usage as a screening test in general population or those with non-specific complaints have not been proposed. [11]

According to the above-mentioned findings, it would be concluded that IB is a sensitive and specific method for the detection of anti-nuclear antibodies and anti-antigens extracted from the nucleus. However, the specificity of this test in the present study was lower than those of ELISA, which may be attributed to the false negative cases in ELISA test. In conclusion, due to high sensitivity and specificity of immunoblotting in detection of anti-nuclear antibodies in rheumatologic patients, it would be used as a screening test and as a routine test to detect specific antibodies against specific nuclear particles.

Figure 1:The gender and disease information of patients.

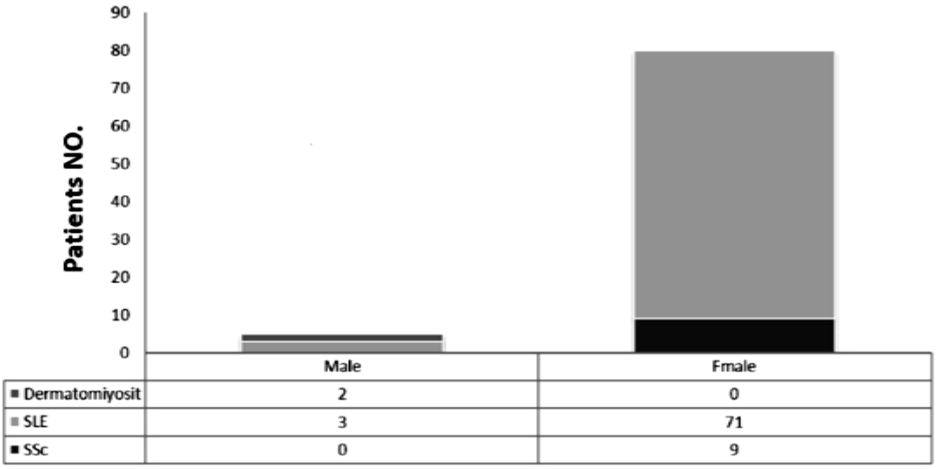

Figure 2. Representative picture illustrating the detection of ANA by IF.

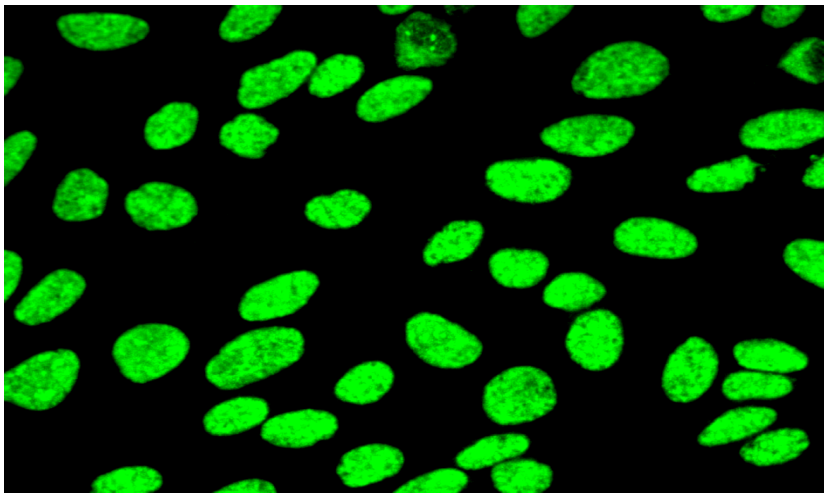

Figure 3. Representative scheme illustrating the detection of ANA by Immunoblotting.

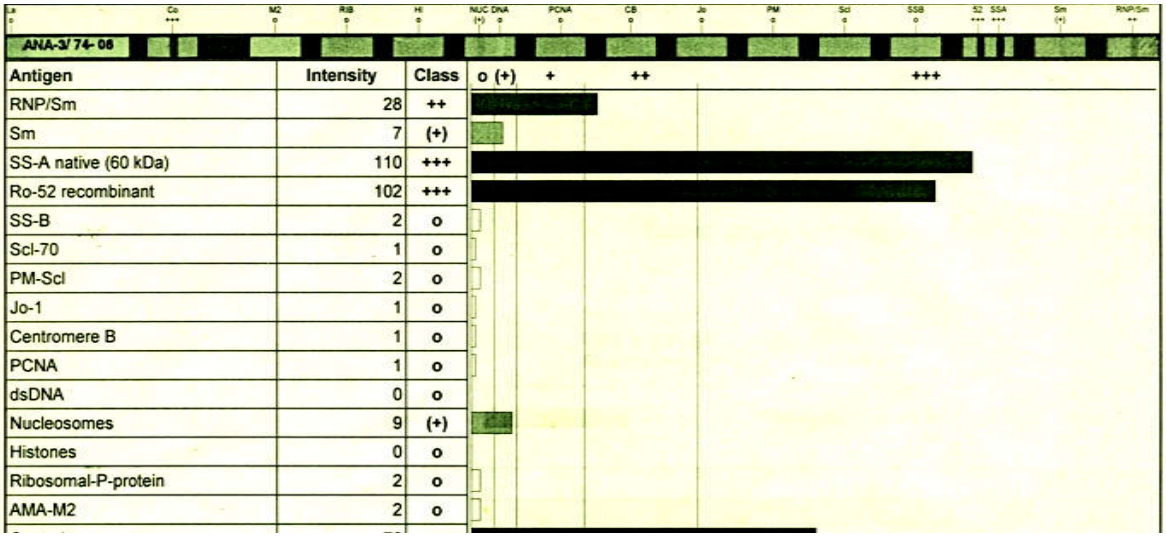

Table 1. Comparison ofIB sensitivity with ELISA and IF methods.

	ELISA- N (%)	ELISA+ N (%)	IF- N (%)	IF+ N (%)
IB+	15 (68.2)	59 (97.3)	1 (9.1)	73 (98.6)
IB-	(31.8)	4 (6.3)	10 (90.9)	1 (1.4)
SUM	22 (100)	63 (100)	11 (100)	74 (100)

ELISA: enzyme-linked immunosorbent assay, IF:immunofluorescence, IB:immunoblot

Conflict of INTEREST

The authors have no conflicts of interest.

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