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Association of Immunofluorescence pattern of Antinuclear Antibody with Specific Autoantibodies in the Bangladeshi Population

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Abstract

Antinuclear antibody (ANA) is useful in the diagnosis of connective tissue disorder (CTD). Association of specific autoantibodies with the immunofluorescence pattern of ANA in CTD, noted in western literature has been considered as reference in all over the world. However, in Bangladesh no such research work or data correlating the autoantibodies and their ANA patterns is found. Objective of the study was to identify an association between immunofluorescence patterns of antinuclear antibody on HEp-2 cell and more specific antinuclear reactivities (e.g. anti-dsDNA and anti-extractable nuclear antigen) in the serum samples of CTD patients. Serum samples of 152 CTD patients (Systemic lupus erythematosus, Rhumatoid arthritis, Sjogren´s syndrome, Systemic sclerosis, Polymyositis, Mixed connective tissue disease) were diagnosed clinically, attending at Bangabandhu Sheikh Mujib Medical University (BSMMU) during the study period of January, 2010 to December, 2010. Samples were subjected for ANA testing by Indirect Immunofluorescence (IIF) on HEp-2 cell (ALPHADIA) in dilution of 1:40, anti-dsDNA by ELISA and anti- extractable nuclear antigen (anti-ENA) by Dot Immunoblot. Dot blot strips were tested for anti-Sm, anti-RNP, anti-SSA/Ro, anti-SSB/La, anti-Scl-70 and anti-Jo-1. Out of 152 patients 110 (72.3%) cases were ANA positive by IIF on HEp-2 cell. ANA positive sera exhibited four fluorescence patterns such as speckled (50.8%), peripheral (21.6%) ,homogenous (18.1%) and nucleolar pattern (9%). Peripheral pattern and homogenous pattern was predominantly associated with anti-dsDNA ($p<0.05$). Speckled pattern was significantly associated with anti-ENA ($p<0.05$).The most commonly identified antinuclear autoreactivity was directed towards anti-RNP (25.7%) then anti-Scl-70 (20%), anti-SSA (14.2%) and anti-SSB (5.7%). Multiple anti-ENA reactivities were identified in 34.28% cases. Peripheral and homogenous pattern is strongly associated with anti-dsDNA and speckled pattern may predict anti-ENA (specially ribonucleoprotiens). As a definite correlation between the ANA patterns and the group of antibodies was detected by dot immunoblot, one could predict presence of certain specific auto antibodies for a particular ANA pattern identified. This may restrict on the cost of laboratory investigations in a developing country like Bangladesh. Thus, ANA-IIF method may reduce the expense of detailed immunological work-up with minimal loss in diagnostic accuracy.

Introduction

An autoimmune response is a common manifestation of the connective tissue diseases. Antinuclear antibody (ANA) test and tests for specific autoantibodies to nuclear antigen play an important role in the diagnostic evaluation, prognostic assessment and monitoring of patients with autoimmune connective tissue disorder (CTD).[1] The indirect immunofluorescence (IIF) performed on cultured human epithelial (HEp-2) cell is currently considered to be the gold standard for the detection of antinuclear antibodies (ANA). Positive fluorescence staining indicates the presence of ANA but does not allow precise identification of these autoantibodies. For detection of immune profile, additional techniques such as western immunoblotting, enzyme linked immunosorbent assay (ELISA) or line immunoassay are required.[2-4] Since a characteristic profile of ANA is associated with most CTD, identification of the fine specificity may provide valuable clues to the diagnosis. These are usually inferred using data obtained from western studies. However, no such study has been done in the Bangladeshi population.

In this study we analysed 152 serum samples of CTD referred to our laboratory for ANA testing.

Further identification of the specific autoantibodies were done by ELISA for the detection of anti-dsDNA antibodies and by Dot immunoblot for detection of antiextractable nuclear antigens (anti-ENA antibodies). If a definite association is found between the immunofluorescence patterns of ANA and the fine reactivity of autoantibodies, patterns of ANA-IIF could be use to predict presence of autoantibodies to diagnose a CTD precisely. This would economize on the cost of laboratory investigations in Bangladesh.

Materials and Methods

Sample: A total of 152 serum samples of CTD patients referred by rheumatologists in BSMMU for ANA testing during the period of January, 2010 to December, 2010 were enrolled in this cross sectional study. Blood (5 ml) was drawn from CTD patients i.e Systemic lupus erythematosus (SLE), Rhumatoid arthritis (RA), Sjogren´s syndrome (SS), Systemic sclerosis (SSc), Polymyositis (PM), Mixed connective tissue disease (MCTD) and sera were separated from the clotted blood samples by centrifugation. Sera were stored at 4ºc if testing was planned within 72 hours or at -20ºc for testing after three days (without freezing and thawing). Each of the serum samples was subjected for ANA testing by Indirect Immunofluorescence on HEp-2 cell, Dot immunoblot for anti-ENA and ELISA for anti-dsDNA.

Indirect immunofluorescence on HEp-2 cell: Indirect immunofluorescence was done by a commercially available kit. Serum diluted 1:40 in phosphate buffered saline (PBS) was overlaid onto fixed HEp-2 cell (ALPHADIA, Belgium) for 30 minutes at room temperature. Slides were washed twice for five minutes each with PBS, overlaid with fluorescence labeled conjugate, which is antihuman IgG heavy and light chain specific and incubated for an additional 30 minutes. After washing twice, a coverslip was placed over the slide, and the slides were read using a fluorescence microscope at 40 power.[5] The main fluorescence patterns seen were speckled, homogeneous, peripheral and nucleolar.

Dot Immunoblot: The Dot immunoblot method is a qualitative assay, which utilizes strips of nitrocellulose on which purified antigens are blotted at prelocated spots. Coated antigens used in this study were Sm, Sm/RNP, SSA/Ro, SSB/La, Jo-1 and Scl-70. The antigen sources used are bovine and rabbit thymus (SSA and Sm) or calf spleen and rabbit thymus (SSB and Sm/RNP). The test procedure was performed according to directions supplied by the manufacturer (D-tek, Belgium). Test strips were incubated for 10 minutes with a diluted patient serum in a PBS-Tweeen solution. Subsequently the test strips were washed by gentle agitation in a test tube filled with PBS-Tween for 1 minute. After the excess buffer solution was removed with a filter paper, the test strips were incubated with an alkaline phosphatase-Protien A conjugate for 10 minutes. The test strips were then washed for 1 minute by gentle agitation by PBS-Tween. Again excess buffer was removed with filter paper. Finally the test strips were stained with 5-bromo-4-chloro-3-indolylphosphate for 5 minutes. The reaction was terminated by washing the test strips with deionized water. The strips were then air dried. Only strips on which the positive control position was stained as a clearly marked blue spot were able to be evaluated and used for this study.[6]

ELISA: Antibody against dsDNA was detected by commercially available ELISA kit (Orgentec, Germany). Microwells were pre-coated with calf thymus dsDNA antigen. The calibrators, controls, and diluted patient samples were added to the wells and autoantibodies recognizing the dsDNA antigen bind during the first incubation. After washing the wells to remove all unbound proteins, purified peroxidase labeled rabbit anti-human IgG conjugate was added. The conjugate bounded to the captured human autoantibody and the excess unbound conjugate was removed by a further wash step. The bound conjugate was visualized by TMB substrate which gives a blue reaction product, the intensity of which is proportional to the concentration of autoantibody in the sample. Phosphoric acid was added to each well to stop the reaction. This produces a yellow end point colour, which was read at 450nm.

Statistical analysis: Data analysis was performed using SPSS PC version 10. A Chi-square test was used to identify association. A P value <0.05 was considered significant.

Results

In this cross sectional study, 152 samples of CTD patients (Systemic lupus erythematosus, Rheumatoid arthritis, Sjogren´s syndrome, Systemic sclerosis, Polymyositis, Mixed connective tissue disease) were analyzed who were diagnosed clinically and with relevant laboratory investigations. The study tried to find out the correlation of ANA fluorescence pattern with specific autoantibodies. Among these samples, 110 (72.3%) were ANA positive by IIF method. Four patterns of nuclear fluorescence were noted: the homogenous pattern, in which the entire nucleus

fluoresced (Fig.1); the peripheral pattern, in which the fluorescence is located along the rim of nucleus (Fig.2); the speckled pattern in which the fluorescence is localized as discrete spots in the nucleus (Fig.3); and the nucleolar pattern in which fine and discrete speckles in the nucleolus within a dark border (Fig.4). In these fluorescence positive samples, speckled pattern was the most common pattern seen in 50.9% cases, followed by peripheral pattern 21.8%, homogenous pattern 18.2% and nucleolar pattern 9.1%. The various ANA patterns seen in the IIF positive samples are shown in Table-I.

In comparison with ANA-IIF results of these samples, various combinations of specific auto-antibodies were observed. Table II presents the probabilities of detecting anti-dsDNA or anti-ENA antibodies (anti-SSA, anti-SSB, anti-RNP, anti-Sm or anti-Scl 70) according to staining pattern. The homogenous and peripheral pattern in this series exhibited strong association with anti-ds DNA ($p<0.05$). Speckled pattern shows combination with anti-ENA ($p<0.05$). Nucleolar pattern was observed in 10 samples and were strongly associated with anti-Scl-70.

The most commonly identified anti-ENA was anti-RNP 25.71% which was mostly associated with speckled pattern, 20% anti-scl-70 showed homogenous, speckled & nucleolar pattern, anti-SSA 14.28% and anti-SSB 5.71% was associated with speckled pattern. Multiple specific autoantibodies were found in 34.28% cases which were associated with homogenous, speckled and peripheral pattern.

ANA-IIF negativity was observed in 42 of the total 152 samples under study. Of these, 4(9.5%) exhibited positivity with anti-dsDNA. There was a single case in the entire study that exhibited strong positivity for SSA/Ro but negative for ANA-IIF. However, 16 (14.5%) of the ANA-IIF positive samples showed negativity for specific autoantibody. The ANA pattern observed in these cases were homogenous 2 (12.5%); speckled pattern was seen in 8 (50%) cases and 6(37.5%) cases exhibited nucleolar pattern (Table III).

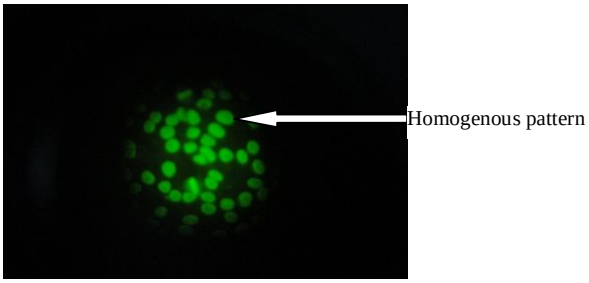

Fig-1: Homogenous pattern on HEp-2 cell

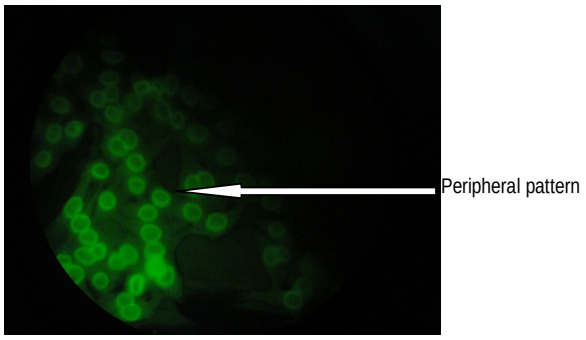

Fig-2: Peripheral pattern on HEp-2 cell

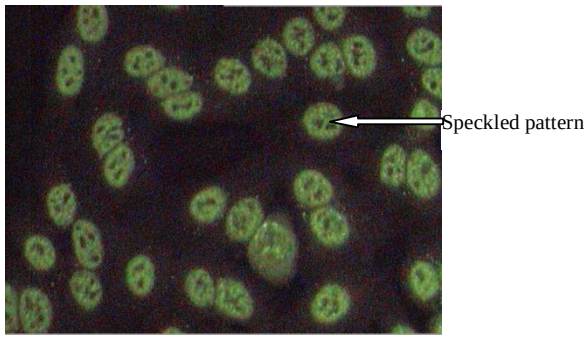

Fig-3: Speckled pattern on HEp-2 cell

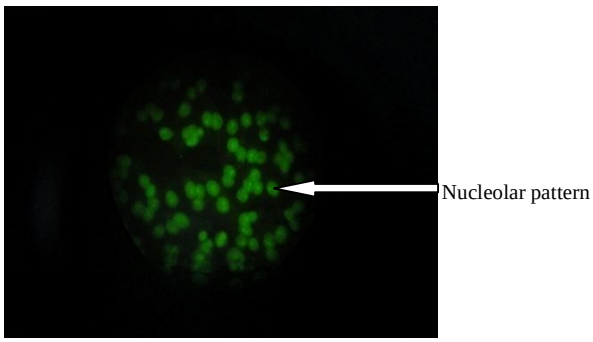

Fig-4: Nucleolar pattern on HEp-2 cell

Table 1: ANA fluorescence staining patterns observed in this study population

ANA Patterns	Samples (n=110)
Homogenous	20(18.2)
Speckled	56(50.9)
Peripheral	24(21.8)
Nucleolar	10(9.1)

Table II: Association of specific autoantibody with ANA-IIF staining pattern of 110 samples

Specific autoantibody	ANA -IIF pattern			
	Homog-enous	Speckled	Peripheral	Nucleolar
Anti-dsDNA n=48	12 (25)	14 (29.1)	22 (45.8)	0
Anti-RNP n=20	0	18 (90)	2 (10)	0
Anti-SSA and or SSB n=16	0	10 (62.5)	6 (37.5)	0
Anti-Sm and or Sm/RNP n=6	2(33.3)	2 (33.3)	2 (33.3)	0
Anti-Scl 70 n=14	6(42.8)	4 (28.5)	0	4 (28.5)

Note: Figure within parenthesis indicates percentage

Table III: Immunofluorescence staining patterns observed in ANA-IIF positive but Anti-ENA negative samples

ANA Patterns	ANA-IIF (+)/ Anti-ENA (-) (n=16)
Homogenous	2 (12.5)
Speckled	8 (50)
Nucleolar	6 (37.5)

Note: Figure within parenthesis indicates percentage

Discussion

Antinuclear antibody testing is usually the first step in the immunologic diagnosis of CTD. Indirect Immunofluorescence on HEp-2 cell is the standard approach for detecting ANAs, and the staining patterns depend on the location of the target antigen. These patterns correspond to the presence of autoantibodies against different nuclear antigens.[7,8] Although some IIF patterns strongly suggest distinct specificities, additional tests are required to demonstrate antibody reactivities against specific nuclear and cytoplasmic antigens. These tests are used to either support the diagnosis (disease specificity) or to identify subsets of patterns that are prone to particular disease manifestation (prognostic marker). Further, the results of the test could be used in patterns with a wide differential diagnosis, so that the results of the tests may exclude systemic autoimmunity (negative predictive value) or may assist the diagnostic process by meeting the diagnostic criteria of a particular rheumatic disease (disease specificity).[9]

However, there is no consensus about the best way to identify the fine specificity of ANA. We report here on the probability of detecting ANA in a cohort of patient serum samples referred for ANA testing; and identifying specific antinuclear reactivities (anti-dsDNA and anti-ENA antibodies) in ANA positive serum samples, using ELISA and Dot Blot respectively. The study that most closely resembles ours, reported in 2010, by Sebastian *et al*.[10] This study reports the results of sera tested on ANA using HEp-2010/ liver biochip and a screening dilution of 1:100. In another study by Slater and Shmerling, ANA was performed on HEp-2 cell substrate at a titre of 1: 40.[11]In Albania, Sulcebe and Morcka also reported a similar study in 1992.[12] They observed the results of sera tested on ANA using rat liver substrate and a screening dilution of 1:100 on rheumatic as well as non-rheumatic diseases.

In this study, 72.3% serum samples were positive for ANA by IIF of which speckled pattern 50.8% was the most common pattern followed by peripheral pattern 21.64%, homogenous 18.1% and nucleolar pattern 9%. Peene *et al*., 2001 reported that the most prevalent fluorescence pattern was speckled 42.5%, followed by homogeneous 41.4% and nucleolar 10.6% in their study.[5]

The speckled pattern, frequently identified ANA pattern in this study showed an association with Sm, RNP, SSA/Ro or SSB/La. Thus, with a speckled pattern one could project further that the serum had antibodies against anti–Sm, RNP, SSA or SSB. Mutasim and Adams also reported similar association between speckled pattern and various ribonucleoprotien in their study.[13] In this study, anti-dsDNA antibodies are almost exclusively identified in serum samples with homogeneous and peripheral ANA staining. Anti-ENA antibodies are more randomly distributed between different fluorescence patterns. It should be noted that the fluorescence pattern may result from the simultaneous occurrence of different ANA. Nucleolar ANA pattern showed association with anti-Scl-70.

Positive ANA-IIF with negative immune profile was noted in 14.5% of cases. A positive result with ANA-IIF, together with negative results in line immunoassay, was also noted earlier and attributed to the presence of anti-dsDNA antibodies. Vos *et al* have found ANA positive samples negative with line immunoassay but found positive for anti-dsDNA antibodies.[14]

In this study, 4 samples were ANA-IIF negative. However, they had positive anti-dsDNA. This could be explained by that antibodies tend to rise during flares of disease.[5] There was only one case which was positive for anti-ENA by Dot immunoblot, was negative by ANA-IIF. This serum showed anti- SSA positivity; however a single case is insufficient to give any explanation. Hoffman *et al* observed that line immunoassay is more sensitive for the detection of SSA/Ro-52 than ANA-IIF even when Hep-2000 cells are used as SSA is a cytoplasmic antigen.[15] Screening with ANA-IIF may miss anti Scl-70 and anti jo-1 antibodies, which is relevant for systemic sclerosis and polymyositis. Scl-70 and Jo-1 reactivity may be undetected or unreported with ANA-IIF as these antibodies give a cytoplasmic positivity rather than nuclear staining pattern on IIF and that ANA could have been reported as negative. So that cytoplasmic staining pattern identification is also important in selective cases when ANA-IIF is reported "negative".[10]

In conclusion, Immunofluorescence pattern could predict the presence of certain specific antibodies in the sera. These correlations are of relevance for the diagnosis of disease more accurately. It may be used for screening purposes for patients with autoimmune disease in daily clinical practice.

Decision can be taken from ANA-IIF result, which specific autoantibody to be tested from immune profile and that will be cost effective. Cytoplasmic pattern in ANA-IIF is also an important tool like nuclear pattern. Dot immunoblot may be considered as a useful investigation particularly for clinically suspected cases with unusual presentation or patients with overlap syndrome.

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