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Immunology

DETECTION OF ANTI-NUCLEAR ANTIBODIES INDIRECT IMMUNOFLUORESCENCE VS IMMUNOBLOT

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ABSTRACT

Aim: Detecting Anti- nuclear antibodies (ANA) Indirect Immunofluorescence or Immuno blot which is more specific and sensitivity help in diagnosis & Prognosis. **Method and Result:** Patient attending OP/IP in tertiary care hospital of 100 cases, excluding criteria ruled out other disorders showing similar symptoms, including criteria both male and female patient age group above 18yrs. All samples are sent for IIF and immunoblot detection test and out of 100, where only 35 positive with both IIF and immunoblot positive , 65 cases IIF negative but immunoblot positive with titters higher then 1+ for more than one antigen. **Discussion:** Indirect Immunofluorescence method reported in titre, highest dilution antibodies 1:100 is positive for auto immune disorders. IIF technique required skilled operators, detecting on monolayers of cultured epithelial cells. It tells presence of ANA but not specific antibodies clearly. In contrast, Immunoblot uses specific antigens attached to a solid surface to detect antibodies in patient sample. When specific antibodies are present, they bind to the antigen on the membrane, resulting in a visible dark line. This method is more specific, easier to train personnel and more cost-effective. Additionally, long term analysis of immunoblot pattern can offer important insights into disease prognosis. **Conclusion:** Diagnosis & prognosis of ANA is easier with Immunoblot detecting specific antigen then indirect immunofluorescence method which required high technology and skilled personal and less prognostic value.

KEYWORDS : Indirect immunofluorescence, Immunoblot

INTRODUCTION

Anti- nuclear antibodies (ANA's) also named as antinuclear factor (ANF) are antibodies produces by human immune system which bind to cell nucleus. When human body expose to foreign proteins called antigens is known as antibodies, some cases antibodies produces to human antigens known as autoantibodies, of which ANAs are one of type autoantibodies.[1]

ANA antibodies can be divided into two main types: one type binds to nuclear material, and the other targets histones and DNA. Antibodies that bind to histones are called anti-histone antibodies, while those that bind to DNA are referred to as anti-dsDNA antibodies. Other types of antibodies that affect nuclear antigens, and were the first to be detected, are anti-Smith antibodies[2]. Other varieties include anti-SSA/Ro, Jo-1, anti-U3-RNP, anti-SSB/La, Scl-70, and anti-centromere antibodies [3,4].

A variety of ANA subtypes can be found in different disease conditions.

1. Nucleus binding different proteins or protein complexes are Anti-Sp100, Anti-centromere antibodies which are one of the subtypes of ANA antibodies.
2. Antibodies that bind to nuclear pore complexes include anti-dsDNA antibodies, anti-histone antibodies, anti-Ro antibodies, anti-Sm antibodies, anti-La antibodies, anti-nRNP antibodies, and anti-Scl-70 antibodies, which are considered a second category of ANA antibodies.

Autoimmune disorders like systemic lupus erythematous [5, 7], Sjogren syndrome [6], mixed connective disorders [7] rheumatoid arthritis or scleroderma[8] can be diagnosed using ANAs concentration.

When we order ANA test?

Consulting Physician suspect to order ANA test when patient present with symptoms of Muscle pain, fever, blisters, rash, changes in skin colour, joint pains, stiffness, fatigue, and swellings[5,6,7,8]

How we test for ANA

Patient blood serum is used to detect ANA autoantibodies concentration .Common quantifying gold standard test is 1. indirect immunofluorescence (IIF)[9] patient blood sample is mixed with special tissue matrix known as substrate Hep-2 cell which have strong affinity for auto immune antibodies ANA if present in patient sample, other method is 2.Enzyme linked immunosorbent assay (ELISA) in which 10 different cytoplasmic and nuclear antibodies are determined in single process reaction test with optional of antibodies verity. Next easiest method is 3. Immuno blot in which we detect individual antigen on antigen coated strips, at a time single strip are coated with different antigens so can detect 15, 28, or 34, antigens specifically.[15]

METHOD

Patient attending to OP/IP in tertiary care hospital with symptoms of Muscle pain, fever, blisters, rash, changes in skin colour, joint pains, stiffness, fatigue, and swellings are taken in this study total of 100 cases, excluding criteria where ruled out other disorders showing similar symptoms, including criteria both male and female patient age group above 18yrs.

The patient sample are studies by both methods IIF method patient blood sample is mixed with special tissue matrix known as substrate Hep-2 cell which have strong affinity for auto immune antibodies highest dilution serum at which antibodies is detectable titre higher than 1:100 are taken as positive, and second method Immuno blot by Euroline company of 15 strips kit which is used to detect 15 types of different nuclear, cytoplasmic and mitochondrial antigens in a multiplex approach for detecting of these antibodies in a single reaction.

The principle of testing is the test strip is coated with parallel lines of purified highly specific antigens; first step of reaction immune blot strip is incubated with patient diluted sample. Sample with positive autoantibodies binds to corresponding antigen site. Antibodies bound are detected through second incubation by enzyme labelled anti-human IgG (enzyme conjugate) which is catalysed by colour reaction.

Ethics:

Patient concern is taken during consultation that there data will be used for academic and research purposes, The Hospital academic committee has approved to use of data for academic purposes.

RESULT

Sample showed following results by IIF and immune blot detection out of 100, where only 35 cases showed titter above 1: 100 positive with both IIF and detected specific high titter antibodies attached in Euroline strip immune blot positive, 65 cases showed IIF titter (<1:100) negative but immune blot Euroline strip showed positive with titters higher then 1+ for more than one antigen.

Significance of Positive Test Clinically

3 to 5 percent of the general population are effected by Autoimmune disorders and among them ANA test is one of the specific tests used to diagnose these conditions.

In the general population, systemic autoimmune disorders affect between 3 to 5 percent of people, and one of the specific testing methods for detecting these diseases is the estimation of ANA [10].

When a person shows symptoms of an autoimmune disorder, ANA testing is often the first test performed. If the IIF method results are positive, further testing with the immunoblot method is done to

identify specific autoantibodies. A notable clinical correlation exists in subtypes of ANA and autoimmune connective tissue disorders. However, these tests do not always confirm that a person has or will develop an autoimmune disease. Serval autoimmune diseases can be detected by presence of ANA in patient serum. A negative ANA result using the IIF method is expected in certain specific inflammatory conditions such as Ankylosing spondylitis [11].

DISCUSSION

IIF-ANA method patient sample when tested the patient blood sample is mixed with special tissue matrix known as substrate Hep-2 cell which have strong affinity for auto immune antibodies ANA if present in patient sample. Then a special fluorescent dye is attached to another antibody which will attach to ANA.

Next under fluorescent microscope skilled and trained doctor or technician are able to see different fluorescent patterns when presence of ANA positive. Some low positive patterns are seen in certain diseases. This give the observing person idea and type of ANA present in blood which has to be further testing's. Patterns seen are Homogenous, Speckled, and Centromere Nucleolar.[12]

In Indirect Immunofluorescence method autoantibodies levels are reported in titre, 1:40/1:80 are reported as low positive titres, clinical significance increase as the titres are high (>1:160), low positive (≤1:160) can be seen in healthy population around 20% common among elderly age group. Only around 5% of the healthy population have ANA titres of 1:160 or higher which is highest dilution serum at which antibodies is detectable titre higher than 1:100 is regarded as positive for auto immune disorders. [9] In IIF technique ANA are detected on monolayer of cultured epithelial cells which is time consuming and required trained skilled technicians or lab doctors. This method only tells presence of ANA but not specific antibodies clearly. [Table 1]

Table 1: Patterns Observe in Indirect Immunofluorescence and Corresponding Antigens, in Diagnosis[13]

Patterns	Related Antigens	Related Diagnosis
Centromere	Kinetochore: CENP-A,CENP-B,CENP-C,CENP-F	SSc (limited)
Peripheral/rim or nuclear envelope	Lamins, LAP1/2, gp210, nucleoporin p62; Tpr (nuclear envelope and nuclear pore complex antigens)	SLE, RA, PBC, myositis, autoimmune liver disease, PAPS
Centrosome/centriole (formerly spindle apparatus)	Enolase, ninein, pericentrin	SSc, Raynaud's phenomenon, inflammatory disease
Discrete speckled	Endosome (early endosome antigen 1), GW/P bodies, multivesicular bodies/lysosomes	Neurological conditions, SS, SLE, RA, PBC
Cytoplasmic fibers	Actin, cytokeratin, tropomyosin, vimentin	CAH, DM, infections and other inflammatory diseases
Homogeneous	DNA, histones, chromatin/ nucleosomes	SLE, drug-induced SLE, JIA
Fine speckled	Jo-1, SRP, PDH (mitochondria)	Myositides, DM, PBC, interstitial lung disease
Nucleolar clumpy	U3-RNP (fibrillarin)	SSc
Golgi complex	Golgi proteins	SLE, SS, RA, overlap syndromes, cerebellar ataxia
Dense fine speckled	DFS70/LEDGF-P75	Healthy subjects and other inflammatory conditions
Diffuse homogeneous (nucleoli positive)	Ribosomal proteins	SLE
Nucleolar speckled	RNA polymerase (I to III)	SSc
Coarse speckled	U1-snRNP, U2-6 snRNP (Sm), nuclear matrix	MCTD, SLE, Raynaud, SSc, SS, UCTD
Multiple/ few nuclear dots	Sp100/140, PML bodies, NDP53, p80-coilin, PML bodies	PBC, CAH, SS
PCNA	Proliferating cell nuclear antigen (PCNA), elongation factor of DNA polymerase δ	SLE, lymphoproliferative diseases, SS
Nucleolar homogeneous	PM/Scl, RNA polymerase, To/Th , B23 phosphoprotein/ numatrin	SSc, myositis, overlap myositis/SSc
Diffuse speckled with "cloudy" mitoses	Topoisomerase-I	SSc

Immuno blot method present study we used Euroline ANA profile 3 of 15 test strip which is used to detect 15 types of different nuclear, cytoplasmic and mitochondrial antigens in a multiplex approach for detecting of these antibodies in a single reaction.

The principle of testing is the test strip is coated with parallel lines of purified highly specific antigens; first step of reaction immune blot strip is incubated with patient diluted sample. when patient sample has autoantibodies positive there bind to corresponding antigen site. The bound antibodies are then detected in a second incubation using an enzyme-labelled anti-human IgG (enzyme conjugate) which catalysis a colour reaction.

Each EUROLINE test strip includes a control band that indicates whether the incubation steps were performed correctly.The evaluation is done automatically with the EURO Line Scan software. [14]

Table: 2 EUROIMMUN Recommends Interpreting Results Based on the Signal Intensity.

Signal visual evaluation	Signal intensity on scanner	Result	
No signal	0-5	0	Negative
Very weak band	6-10	(+)	Borderline
Medium to Strong band	11-25 or 26-50	+,++	Positive
Very strong band with intensity comparable to control band	>50	+++	Strong Positive

Results in borderline range (+) should be re-evaluating as increase incidence of negativity is common.[15]

In immunoblotting, antigens are coated on membranes as a solid phase to detect specific antibodies in patient samples.

If specific antibodies are present, they bind to the membrane-bound antigens, and a dark line appears at the corresponding antigen location. A single strip can be coated with different antigens as needed, with products available containing 15, 28, 32, etc., antigens. This method is cost-effective, easy to train personnel, and offers more specificity. Immunoblot patterns have proven to be helpful in long-term follow-up and have prognostic importance.

Supportive Study

Detecting of antinuclear antibodies (ANA) by indirect immuno-fluorescence (IIF) similar patterns are seen for different antibodies which are corresponding to different antigens to verity of autoimmune disorders. To rectify this problem we used immuno blotting technique where different total antigens from Hep-2 cells (TA-Hep-2-C) are coated on strip which provides more specificity and sensitivity then IIF. There are single strip coated with 40 antigens representing different antibodies binding bands which are unrelated to fluorescence patterns of ANA. Immuno blot specific antibodies of many types in a single trip test. This gives immuno blot more diagnostic value. [16]

The use of reference sera allowed the identification of previously described antigens relevant to these diseases. Some patients showed bands that have not yet been reported and may have clinical significance of their own or may be with combination of other autoantibodies. Long-term follow-up of these immunoblot patterns could be of prognostic importance.

CONCLUSION

The above study say detection of ANA in diagnosing or prognosis point of view Immunoblot method is much useful, less cost, easily

technique and software in detecting specific antigen with titters then Indirect immunofluorescence method which required high technology microscope and skilled personal and less prognostic value.

There should be a bigger study with different presentation to prove the accuracy and use of immunoblot then IIF method for diagnosing and prognostic importance of ANA.

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