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Review Article

Microbiology Section

An Overview of Various Diagnostic Methods to Detect Antinuclear Antibodies of Connective Tissue Diseases

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ABSTRACT

Antinuclear Antibodies (ANA) are present in many autoimmune disorders and these disorders are collectively called as Connective Tissue Diseases (CTD). There are various CTDs which includes Systemic Lupus Erythematosus (SLE), Sjogren's syndrome, Systemic Sclerosis (SS), Inflammatory Myositis (IM), Mixed Connective Tissue Disorder (MCTD) and Rheumatoid Arthritis (RA). Detection of ANAs in these CTDs is highly sensitive and is of utmost importance. The ANAs specific to SLE includes anti-double stranded Deoxyribonucleic Acid (anti-dsDNA), single stranded DNA (ssDNA). Scleroderma or Systemic Sclerosis (SS) is an immune mediated rheumatic disease where autoantibodies like topoisomerase 1, Ribonucleic Acid (RNA) polymerase 1 and fibrillarin are useful in diagnosis. Idiopathic Inflammatory Myositis (IIM) such as polymyositis and dermatomyositis are characterised by the presence of autoantibodies like PM-scl (Polymyositis-Scleromyositis), Mi-1 (Myositis specific autoantibody found in idiopathic inflammatory myositis), Mi-2 and Ku (DNA Binding Protein in dermatomyositis). Antibody titres against polypeptides on the U1 Ribonucleoprotein (U1RNP) is useful in the detection mixed CTD. Sjogren's syndrome is characterised by the presence of serum autoantibodies against two ribonucleoproteinic complexes like Ro/SSA (Extractable nuclear antigen found in Sjogren's syndrome related antigen A auto antibodies) and La/SSB (Extractable nuclear antigen found in Sjogren's syndrome or lupus erythematous). ANA analysis can be done by techniques like indirect immunofluorescence method, Enzyme Linked Immuno Sorbent Assay (ELISA), Immunoprecipitation in agar and western blotting. All these diagnostic methods give precise identification of these antibodies with high accuracy. Farr assay, Multiplex Immunoassay (MIA), Flow cytometry, antigen microarray is also gaining importance in the diagnosis of ANAs. The objective of this study was to discuss various methods of ANA testing so that the clinicians know its relevance in diagnosis. Certain advancement in the diagnosis of ANA is also included in this review.

Keywords: Antigen microarray, Enzyme linked immuno sorbent assay, Indirect immunofluoresence, Systemic lupus erythematosus

INTRODUCTION

The Connective Tissue Diseases are classified under the group of autoimmune disorders. These groups of disorders are identified by the presence of ANA. The detection of ANA in these connective tissue disorders is highly sensitive and is of utmost importance. In 1948, bone marrow of SLE patients shows the presence of LE cells, led to the importance of ANA testing [1]. The LE cell is an antibody against deoxyribonucleoprotein which phagocytose the antibody sensitised nuclei with help of neutrophils [1]. In 1950, various antigen antibody immune system was noted in many CTDs for example, nuclear ribonucleoprotein in MCTD, Sm antigen in SLE [2]. ANA autoantibodies can bind specifically and destroy certain structures in the nucleus of the cells. Very few amounts of these antibodies are found in normal population as well and if there is a high rise in titres, then it is suggestive of CTD [3]. Identification of autoantibodies especially the antidsDNA or antismith (anti-sm) antibodies are associated with SLE, anti-topoisomerase-I (anti-scl70) and anticentromere antibodies with SS and anti-Jo-1 with polymyositis.

Anti Sjogren's-syndrome-related antigen A autoantibodies, also called antiSSA/Ro and antineutrophil antibodies antiSSB/La autoantibodies have a broader spectrum of association with Sjogren's syndrome, lupus (systemic and cutaneous form), scleroderma and RA. AntiRNP antibodies have been associated mainly with the MCTD, but may also be found in other rheumatic CTDs [4]. Thus, their detection with high sensitivity and specificity is of utmost importance. The aim of the review was to identify the ANA of CTDs by various methods of detection.

CTD AND IT DETECTION METHODS

Systemic Lupus Erythematosus (SLE)

In 1941, SLE was identified as the most common CTDs by Klemperer, Pollack and Baehr [3]. SLE affects both the sexes; however it is diagnosed in approximately 20 to 150 persons per 100,000 populations [5]. It is a predominant autoimmune disorder that affects females of age group 16 to 55 years in the ratio of 9:1 (female: male 9:1 ratio). The manifestations of SLE may be mild, moderate or severe. The active form of the disease shows increased levels of anti-dsDNA antibodies at a higher proportion and other markers like reduced complement levels of C3 and C4. The decrease in complement levels is due to immune complex deposition and it indicates the severity of the disease [6]. It is a chronic disorder that leads to the deposition of autoantibodies which are stimulated by the hyperactive B-cells and immune complexes at various organs like skin, kidney. These depositions target the nuclear components of the cell [5]. The ANA specific to SLE includes anti-dsDNA, ssDNA. Other antibodies include antiRo, antiLa and antiSm antibodies [5]. In case of drug induced lupus, antihistone antibodies are the hallmark for diagnosis. Though the presence of ANA antibodies in patients serves as the hallmark of the disease with 98% sensitivity and 75% specificity, it may be present in certain conditions like malignancies, hepatic diseases, and unaffected lupus family members and even in 14% of healthy individuals. Therefore, the American College of Rheumatology (ACR) says that the testing of ANA specific autoantibodies is the diagnostic criteria only when a positive ANA testing correlates with clinical suspicion. Specificity of anti-dsDNA in SLE patients is about 92-96% and they form the criteria for SLE classification as per ACR and by Systemic Lupus International Collaborating Clinics (SLICC) [7].

Rheumatoid Arthritis (RA)

This autoimmune disorder shows the involvement of joints leading to synovitis which is followed by severe joint destruction. RA is found to be affected about 0.8% of the total population [7]. Extra articular manifestations are also seen in RA [8]. Prompt and early diagnosis of the RA gives a better outcome for the patients. The early treatment with Disease Modifying Antirheumatic Drugs (DMARDS) makes the prognosis good. Other than Rheumatoid Factor (RF), one more important diagnostic antibody is anticitrullinated protein antigens (antiCCP). Strong genetic association of the allele HLA DR-B1 is seen in seropositive RA patients. A study says that cigarette smoking has a strong association with HLADR-B1seropositive RA patients [9]. AntiCCP antibodies target arginine residues and replace it with citrulline. This antiCCP antibody is more significant in RA patients along with RF for the diagnosis. AntiCCP has 67% sensitivity and 95% specificity and RF which is directed against fragment crystallizable (fc) portion of IgG class of autoantibodies has 69% and 85% as sensitivity and specificity [10]. Because of higher sensitivity and specificity of antiCCP in recent days, RA diagnosis depends on detection of both RF and antiCCP.

Systemic Sclerosis (SS) or Scleroderma

Scleroderma manifests as thickening and fibrosis of skin and other internal organs. CREST syndrome or limited SS which includes conditions like calcinosis, Raynaud's phenomenon, Oesophageal dysmotility, Sclerodactyly and Telangiectasia and severe form of Fibrosis syndrome, oesophageal dysfunction, sclerodactyly, telengectasia and severe form of fibrosis. Life threatening complications of SS includes pulmonary arterial hypertension, interstitial lung disease and kidney involvement [6]. This disorder may be limited to a particular organ and the autoantibody against centromere identify the limited sclerosis whereas autoantibodies such as Topoisomerase 1, RNA polymerase 1 and fibrillarin are useful in diagnosing the diffuse form of the disease [11].

Polymyositis and Dermatomyositis

Polymyositis and Dermatomyositis are actually IIM characterised by the presence of autoantibodies like PM-scl, Mi-1, Mi-2 and Ku. Antibodies against jo-1 can also be found in the disease [12].

Mixed Connective Tissue Diseases (MCTD)

MCTD which is an overlap of the features of SS, SLE and Polymyositis, was identified in late 1970. MCTD is characterised by the presence of pulmonary hypertension, oesophageal dysfunction, membranous and mesangial nephropathy. High antibody titer against polypeptides on the U1 Ribonucleoprotein (U1RNP) correlates with the activity of the disease [13,14].

Undifferentiated Mixed Connective Tissue Disease (CTD) or Overlap Syndrome

The diagnosis of MCTD depends on the presence of very high titer of ANA and anti U1-RNP antibodies. MCTD is an overlap of SLE, scleroderma, myositis, and RA and all the symptoms occur in an orderly fashion rather than simultaneous involvement. These patients may have overlap of symptoms such as Raynaud's phenomenon, myalgia, arthralgia, a positive ANA antibody test. So, this condition is called as either Undifferentiated Connective Tissue Diseases (UCTD) or 'overlap syndrome'. Various autoantibody present in UCTD are Anti U1-snRNP Ab in MCTD (SLE+ myositis+ scleroderma+ RA), Anti PM-Scl Ab in conditions like Myositis + Raynaud's, arthritis, Anti SSA/B + RF+ Anti CCP Ab in association with RA + Sjogren's syndrome [6].

Sjogren's Syndrome

Sjogren's syndrome is characterised by focal lymphocytic infiltration of the salivary and lacrimal glands leading to dryness of the mouth and eyes [6,15]. There is a remarkable B-cell hyperactivity leading to hypergammaglobulinemia and presence of serum autoantibodies against two ribonucleoproteinic complexes like Ro/SSA and La/SSB [15]. Both Ro/antiSSA and La/antiSSB antibodies found to be positive in 70% of patients of Sjogren's syndrome [6].

Significance of ANA Antibodies

Detection of autoantibodies against the cell nuclei plays an important role in diagnosis of various CTDs. [Table/Fig-1] represents the prevalence of ANA in various CTDs such as SLE, MCTD, RA and Sjogren's syndrome [16-28].

ANA positive disorders	Antibodies detected	Prevalence in %
SLE	antidsDNA antiSm Antisn RNP Nucleosomes	95-100
Drug induced SLE	Antihistone	100
Mixed Connective Tissue Disease (MCTD)	U1-nRNP SsRNA	100
Systemic Sclerosis (SS)	Anticentromere Antitopoisomerase (Scl-70)	60-80
Sjogren's syndrome	AntiRo AntiLa	40-70
Inflammatory myositis	AntiPM-Scl AntiSRP Antisynthetase	30-80
Rheumatoid Arthritis (RA)	U1Nrnp ssRNA Histones	30-50

[Table/Fig-1]: Prevalence of ANA in various Connective Tissue Diseases (CTD) [16-28].

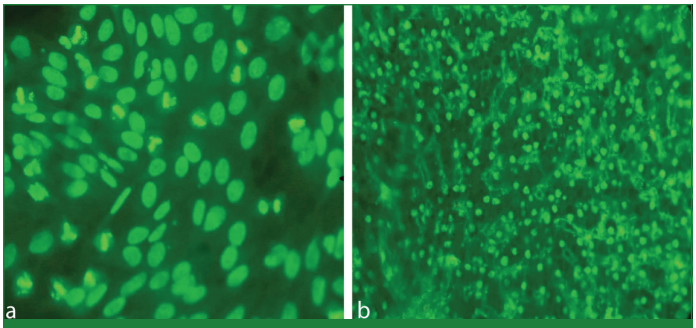
Methods of Detection

Antinuclear antibody analysis can be done by Indirect Immunofluorescence Method, ELISA and other techniques like immunoprecipitation in agar, western blotting. All these diagnostic methods give precise identification of these antibodies with high accuracy [29]. Certain newer techniques like Farr assay, MIA Flowcytometry, Antigen microarray aids in the diagnosis of ANA. Indirect Immunofluorescence method on Hep-2 cell substrates was considered as the gold standard test for ANA detection as said 10 years ago by the ACR [29]. ELISA method is now being widely used in many laboratories as an alternative method to detect ANA by the manual Indirect Immunofluoresence by overcoming its limitations [29].

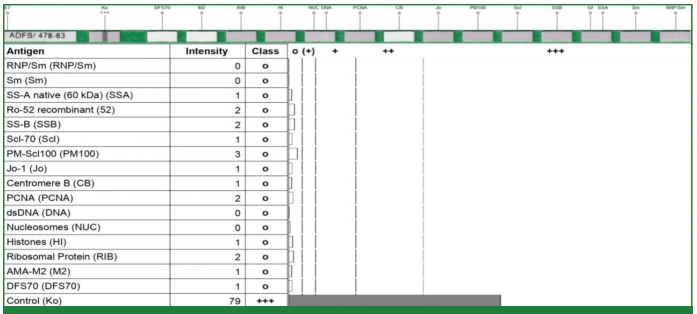
Indirect Immunofluoresence

The detection of ANA by Indirect Immunofluoresence remains as the gold standard test. In this detection method patient's serum is diluted and is incubated with a monolayer of cells which are fixed and permeabilised. If the autoantibodies is present in patients sera, it gets fixed to the cells and this can be identified with anti-human immunoglobulin reagent conjugated to fluorescent tag [30]. Indirect Immunofluoresence is the most sensitive, reliable and simple method for screening of ANA. Human epithelial cell lines-2 (Hep-2) and the primate liver are the two substrates that are commonly used in Indirect Immunofluoresence to which patients sera is added at varying dilutions. The interpretation of the IF-ANA results must always be correlated with the patient's symptoms and signs and that shows the importance of detection of ANA. The disadvantage of the Indirect Immunofluorescence is that it is more time consuming and depends on highly trained personnel [30].

By using the fluorescent microscope one can observe different patterns like homogeneous, rim or speckled if they are nuclear. Nucleolar forms are seen as discrete speckled, grainy speckled and clumpy staining [29,30]. These patterns show their disease associations. If Hep-2 cells are used as substrate then the chances of false-negative are considerably minimised. Fluorescence intensity pattern is expressed as qualitative values of + to ++++ and is to be known to be directly proportional to antibody concentration and indicates the severity of CTD [3]. [Table/Fig-2] illustrates the homogeneous pattern of immunofluoresence in which antigens associated are dsDNA, ssDNA, nucleosomes and histones. Diseases usually associated with this pattern are SLE, drug-induced SLE and juvenile idiopathic arthritis [31-33].



[Table/Fig-2]: a) Homogeneous pattern in Hep 2010; b) Homogeneous pattern in primate liver.



Antigen	Intensity	Class
RNP/Sm (RNP/Sm)	0	o
Sm (Sm)	0	o
SS-A native (60 kDa) (SSA)	1	o
Ro-52 recombinant (52)	2	o
SS-B (SSB)	2	o
Scl-70 (Scl)	1	o
PM-Scl100 (PM100)	3	o
Jo-1 (Jo)	1	o
Centromere B (CB)	1	o
PCNA (PCNA)	2	o
dsDNA (DNA)	0	o
Nucleosomes (NUC)	0	o
Histones (HI)	1	o
Ribosomal Protein (RIB)	2	o
AMA-M2 (M2)	1	o
DFS70 (DFS70)	1	o
Control (Ko)	79	+++

[Table/Fig-3]: Result oriented graph.
This diagnostic test is performed in microbiology lab, Government Medical College and ESI Hospital, Coimbatore using EUROIMMUN kits

Immunodiffusion

Immunodiffusion method follows the principle of Ouchertlony technique of double diffusion in agar rose gel. Rabbit tissue extract or the calf tissue extract along with Extractable Nuclear Antigens (ENA) are there in the well adjacent to patients' serum well. Precipitation bands are formed after a period of 24-48 hours of incubation when the antigen and antibody reacts [21]. This immunodiffusion technique has lower sensitivity when compared to other methods of detection as it detects only less number of antibodies for antigens such as snRNP, Ro, La, Ku, Topoisomerase 1, PM/ Scl [34]. For detection, larger amount of antibodies are required which serve as a major drawback of this method.

Counter Immunoelectrophoresis

This method is very similar to immunodiffusion technique but it overcomes the drawback of immunodiffusion by requiring only lesser amount of antibody and can detect acidic antigens also [22].

ELISA

ELISA technique is another easier method to detect ANA where the multiwall plates are coated with antigens derived from cell nuclei or proteins. ELISA is also called as antigen specific assay where multiple antigens are coated on to the microtitre plates like Jo-1, scl-70, dsDNA, SS-A/Ro, SS-B/La etc., [3]. Patient's serum sample is added to the wells at varying dilutions. If antibody present in serum, it binds to the coated antigen. Then there is addition of antihuman immunoglobulin reagent with an enzyme tag. Quantification of the autoantibody if present is determined by calorimetric method by the addition of the substrate to wells. The intensity of the colour generated is proportional to the amount of IgG specific antibody in sample. Compared with other immunoassay methods, ELISA is featured with higher sensitivity, specificity and less time consuming [30]. These advantages make ELISA to be a very popular choice for researchers from various areas [29].

Immunoblot

Detection of autoantibodies for various CTDs by indirect immunofluorescence may sometimes produce similar pattern. To overcome this problem, immunoblotting against total antigen from Hep-2 cells can be done. The analysis of immunoblotting patterns is of great diagnostic value [24]. Here, the nuclear and cytoplasmic antigens are separated by gel electrophoresis and transferred to strips. Patient's serum is added to the strips in various dilutions. Positive band in the strips indicate the detection of specific antinuclear antibody. Conjugate along with anti-human IgG is used. The sensitivity of immunoblot technique to detect anti SS-A/Ro is very less [3]. [Table/Fig-3] represents result oriented graph for the ANA autoantibodies.

Dot Blot Assay

Dot blot assay is easier, cheaper technique which requires only 30 minutes for performing the test but its major drawback is that both sm and RNP antigen are coated in combination so that both cannot be discriminated separately. The assay includes blotting of various antigens at prelocated spots in the nitrocellulose membrane [3]. The antigens used commonly are bovine and rabbit thymus or calf spleen and rabbit thymus. Detection of sm, SS-A, scl -70 is more specific when bovine and rabbit thymus antigen is used. Patients serum is added in varying dilutions and finally nitro blue tetrazolium dye is used which detect the positive as blue dots [35].

Line Blot Assay

As the name suggests, Line blot Immunoassay is a qualitative test, where the antigen antibody reaction appears as lines on nitrocellulose membrane at equal distances. The strips are incubated with buffer containing blocking protein which prevents the non-specific binding that identifies antibody reactivity to antigens and they appear as distinct lines on a membrane. Line blot is also easy to use and requires less processing time and is comparable to ELISA in sensitivity and specificity [36].

RECENT ADVANCES IN DETECTION OF ANA

Multiplex Immunoassay (MIA)

MIA is a newly developed technique which is used to detect multiple ANA at the same time by using luminex bead technology [37]. The bead suspension is provided with polystyrene microspheres conjugated with different antigens and HEP-2 cells. The immuno analyser identifies the beads with the help of their fluorescent dye and the amount of conjugate. This technique is more efficient than conventional ELISA [3].

Flowcytometry

In recent days, flowcytometry technique has been gaining more importance. It is quantitative fluorescent beads based assay and is also called as Reflex ANA. The assay detects the reactions by combination of internal fluorescent signal. Flowcytometry is highly sensitive and cost effective. The major disadvantage of flow cytometry is that it gives only a single result for each analysis [38,39].

Antigen Microarray

Antigen microarray is a nanotechnology technique where the antigens are impregnated on polystyrene. This technique produces light signals and it is captured by a camera based chip reader. This method is also a quantitative assay where the antibodies are detected using calibration curves [40]. The advantage of antigen microarray is that it's cost effectiveness and can be used for detection of novel autoantibodies. [Table/Fig-4] compares various methods of detection with their advantages and disadvantages [3,40-48].

S. No.	Method	Advantage	Disadvantage
1	IF-ANA	High sensitivity and specificity	Time consuming Can give false positive results
2	ELISA	High sensitivity	Expensive
3	Western blot	High specificity	Expensive Time consuming
4	MIA	Detects multiple antibodies at a time Quantitation possible	Expensive
5	Flowcytometry	Cost-effective High sensitivity	Provides single result at a time
6	Microarray	Complete automation High sensitivity and specificity	Not available easily

[Table/Fig-4]: Comparison of methods of detection- advantages and disadvantages.

CONCLUSION(S)

Detection of ANA needs a long run in future. For further development in detection methods, better instruments with ultrasensitive detection, faster turn around time are required. Newer techniques like MIAs and antigen microarrays serve as an alternative to older methods of detection like Indirect Immunofluoresence, ELISA and immunoblot techniques.

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