

Prediction and Evaluation of Diagnostic Assays for Autoimmune Disorders

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Abstract

Autoimmunity is the realisation of Ehrlich's "horror auto toxicus", the activation of cells that, under normal circumstances, are quiescent and unresponsive. Since decades it has been found that more than 40% of human population are found to have autoimmune disease and 50% of the people are found to have at least one type of autoantibody found in the serum. Antinuclear antibodies are hallmark of auto antibodies production in autoimmune disease, patients suspected with positivity in ANA test. So the studies were carried out to detect prevalence of ANA in autoimmune disease patients. 100 consecutive patients attending the Department of Rheumatology at Government General Hospital and 10 healthy controls were taken into the study. Indirect immunofluorescence method which is a gold standard technique is taken for detection and hence in house method on young mouse liver substrate and HEP₂ cell line substrate were performed. The results were analysed and found that HEP₂ cells have more sensitivity and best binding patterns. The same samples are carried out on ELISA, sensitivity and specificity were compared with Indirect Immunofluorescence technique. If screening ANA is positive many specific ENA such as dsDNA, Sm, U₁RNP, Ro (SSA), La (SSB), Jo1 were detected using Ouchterlony method.

Keywords: Antinuclear Antibodies, Mouse Liver Substrate, HEP₂ Cell Substrate, ELISA.

Introduction

The word “auto” is the Greek word for self. Autoimmunity is the failure of an individual to recognize his own constituent parts as self, which allows an immune response against its own cells and tissues, any disease that results from such an aberrant immune response is termed an autoimmune disease. The term “autoimmune disease” refers to a varied group of more than 80 serious, chronic illnesses that involve almost every human organ system. Autoimmune diseases are conditions in which the immune system damages specific organs or tissues that causes systemic ill health. In most of the autoimmune disease it is thought that self-antigens provide the drive, although the trigger may yet to be exogenous. The cause is unknown but few risk factors have been proposed; they are heredity, complement and sex hormone. The various types of autoimmune disease are Systemic Lupus Erythematos, Rheumatoid Arthritis, Systemic Sclerosis, Sjogren’s Syndrome, Juvenile Idiopathic Arthritis, Hashimoto’s Thyroiditis, Graves disease, Polymyositis, Addison’s disease etc.

Antinuclear antibodies (ANA) are directed against components of the cell nucleus such as DNA, histones and ribo-nucleoproteins. Although these antibodies are the serological hallmark of systemic lupus erythematos, they are also present in a number of other autoimmune disease sera. These may belong to any of the immunoglobulin classes such as IgG, IgM, IgA, IgD, IgE. Indirect immunofluorescence is the method of choice for detection of ANA.

Materials and Methods

Materials

100 samples of patients suspected to have autoimmune disease attending Rheumatology department, Government General Hospital and 10 healthy controls were taken. Young healthy mouse obtained from animal house. HEP₂ cell line maintained at virology department. Others chemicals used are Phosphate Buffer Saline pH 7.2, Anti Human Immunoglobulin with FITC conjugate, Glycerol Saline, Liquid Nitrogen.

Indirect Immunofluorescence

It is a tagged immunoassay where the bound human immunoglobulin is traced with anti-human immunoglobulin tagged with a fluorescent dye. Indirect immunofluorescence is widely employed in rheumatology to detect and measure antinuclear antibodies (ANA) in patients’ sera. When suitable substrate expressing the cellular antigen is allowed to react with sera containing specific antibodies, antigen antibody reaction takes place resulting in the deposition of immune complex at the site where the cellular antigen is located. Then the immune complex is traced with fluorescein labeled anti-human immunoglobulin. Different types of patterns of immunofluorescence correspond to specific autoantibodies. Different substrates can be employed depending on the type of antibody which is to be detected.

Methods

Mouse liver substrate

Substrate Preparation

Young healthy mouse was obtained from animal house. Mouse was anesthetized with chloroform and the abdominal cavity was opened via ventral incision. The liver was dissected out and washed well using normal saline. Cut section of liver was made into 3-4mm thick pieces; the cut surface was then rinsed in saline to remove RBCs. The scratch free micro slides were taken. They were washed thoroughly to remove grease. On each slide three circles were marked by using diamond pencil. Liver cells were transferred from the cut surface to glass slides by impression smear. The tissue section slides were placed in staining troughs and liver cells were fixed to slides by adding cold acetone to troughs and kept in refrigeration for 15 minutes. Acetone was decanted after 15 minutes; slides were kept back in the trough & placed in deep freezer at -20°C for further use.

Procedure

Blood samples were taken from 100 patients and transferred to plastic screw capped vials and labelled. The vials containing blood samples were centrifuged at 2000rpm for 5 minutes for serum separation. Patient's sera was then taken and placed in water bath at 56°C for complement inactivation. The complement inactivated sera to be tested were diluted at 1:20 through 1:160 in PBS. The patient's sera and control sera were marked on horizontal rows and vertical columns on the dilution plate. For each patient's sera and control sera which were labelled on the dilution (150×100mm) and added to it 190µl of PBS, pH 7.2. By using micropipette, 10µl of serum was added to its corresponding wells in dilution plate containing 190µl of PBS, pH7.2. This makes 1:20 dilution and mixed thoroughly. Different tips were used for each serum; pipette 100µl of diluted serum of 1:20 dilution from the well and makes the doubling dilution 1:40, 1:80, 1:160. Smooth out wet cloth in the bottom of the trough (humid chamber) and place the labelled slides on the moist chamber with the tissue section facing up. Separate Pasteur pipette were used for each serum dilution series, 50µl of the various dilutions of each serum were added to appropriate sections of labelled slides. After patient's sera and control sera had been added to slides, cover the trough and incubate at 37°C for 20-25 minutes in a moist chamber. After incubation, the slides were placed in troughs and the slides were rinsed with PBS, at pH7.2 for 3 times in 15 minutes and washing was done carefully without distributing the tissue section. Washing was improved by using magnetic stirrer at slow speed. After washing, PBS was drained off from the troughs and excess PBS was wiped off with tissue paper which was done carefully without touching the tissue section. Slides were placed in the humid chamber. FITC conjugate was diluted with PBS in the ratio 1:50. 25µl of diluted FITC conjugate were added to each slides using micropipette and then slides were incubated at 37°C for 20-25 minutes. After incubation, the slides were placed in troughs. Slides were again rinsed with light stream of PBS, pH7.2 for 3 times for removal of unbound conjugate from the section without damaging the tissue section of slides. The washing was done for 15 minutes. PBS was drained off from the trough and excess PBS which was present around each section was wiped off with tissue

paper without touching the tissue section. A drop of glycerol saline was added at the centre of tissue sections and cover slips were placed over the sections. The slides were examined under Fluorescence microscope.

Interpretation

In the evaluation of patient's sera using mouse liver substrate cells, the presence of circulating antinuclear antibodies was well established and was found to be well associated with the autoimmune diseases. IIF-ANA test was positive in initial three doubling dilutions 1:20, 1:40, 1:80 in 92.3% of SLE, 20% of RA, 20% of JIA, 21.42% Systematic Sclerosis, 40% of Sjogren's Syndrome, 100% of MCTD and 25% of polymyositis. Positivity above 1:80 dilution found in 89.78% of SLE, 12% of Ra, 10% of JIA, 14.28% of polymyositis. Out of 10 healthy controls 2 were positive in low dilutions (1:20, 1:40).

HEP₂ cell maintenance

HEP₂ cell substrate

HEP₂ cells were maintained in continuous culture in RPM medium containing 10% fetal calf serum and 2% maintenance medium by subculture every 3-4 days. Multi-spot slides were prepared. Slides were sterilized by using dry heat at 160°C for 1hr. 25ml HEP₂ cell culture containing 5×10^4 cells/ml were dispensed into 90mm diameter Petri dish. Sterile periodically cell count was assessed under an inverted microscope. When cells had grown to confluence, the slide were washed in PSB for 15min fixed in acetone: methanol(1:1) for 15min. Then Slides were rinsed in distilled water for 15min to wash off the fixative, air dry and stored in plastic bag at -20°C until required.

Process description

Patient's Sera was placed in water bath at 56°C for 30 minutes for complement inactivation. Complement inactivated samples were diluted 1/20, 1/40, 1/80, 1/160 in PBS. 50-100µl of diluted sera was added serially to the HEP₂ cell substrate & kept in incubator at 37°C for 20-25 minutes in a moist chamber. After incubation, the slides were washed in PBS in troughs for 3 times. AHIG FITC conjugate (diluted 1/50) in PBS was added on the slides & incubated at 37°C for 20-25 mts. The slides were washed in PBS for 3 times. The slides were covered with a drop of glycerol saline & cover slips applied & seen under fluorescence microscope. However samples were tested at a screening dilution of 1:40 rather than 1:20 since HEP₂ cells were more sensitive and many normals were positive at 1:20 dilution.

Interpretation

ANA on HEP₂ cell substrate with sera dilution ranging from 1:20 to 1:160 were analyzed in different disease groups. The specific patterns in different dilutions were studied. In 1:20, 1:40, 1:80 dilution ANA positivity was found in 97.44% of patients of SLE, 32% in RA, 40% in JIA 35.71% in Systematic Sclerosis, 60% in Sjogren's syndrome, 100% in MCTD and 25% in polymyostis. In 1:160 dilution it was found that 92.3% of patients of SLE, 28% in RA, 40% in JIA, 35.71% in Systemic Sclerosis, 40% in Sjogren's Syndrome, 100% in MCTD and 25% in polymyositis were positive

for ANA. 37% of homogeneous, 26% of speckled, 13% of Rim, 5% of nucleolar and 19% of mixed patterns were observed.

ELISA

ELISA is a type of tagged immunoassay, where antigen-antibody reactions are traced with anti human immunoglobulin tagged with enzyme. ELISA is widely used to detect and measure different types of auto antibodies in the sera of patients. ELISA can be performed on micro plates coated with purified antigens or it can be performed on nitrocellulose strips blotted with fractionated antigens or on strips coated with purified antigens.

Materials Required

- Distilled water, micropipette, disposable pipette tips, ELISA washer, sample diluents, stop
- Solution, conjugate, washes buffer, calibrator, TMB substrate, positive control, negative
- Control, ELISA reader capable of reading absorbance at 450 nm.

Reagent Preparation

Wash buffer is prepared by adding 25ml of wash concentrate to 475ml of distilled water and it is stored at room temperature (18-26).

Process Description

Commercial value antigen coated strips were placed on holder. The samples to be tested were diluted in various dilution of 1/20, 1/40, 1/80, 1/120 using commercially valuable diluents. Then 100µl of control and diluents of sample were added to well and incubated for antigen and antibody reaction, which was done at room temperature for 20 minutes. Wells were washed three times with 300µl of wash buffer. 100µl of TMB substrate was dispensed and incubated for 10 minutes at room temperature. 100µl of stop solution was added to the wells. Optical density values were noted at 450 nm using ELISA reader within 15 minutes. A dual wavelength was recommended with reference filter 600-650nm.

Interpretation

Other alternative technique ELISA which is currently used clinical test in the laboratory because of high specificity when the same samples tested in its technique. It is found to be complicated & time consuming biochemical test. But in this high specificity was obtained only by extraction & purification of test antigen & coupling them to a solid phase. And accompany of antigen (unknown) if present, the antibody will correspond to the accompany antigen leads to the false positive reaction. Hence in ELISA technique there is chance of occurring false positive and costly process. The advantage of ELISA is more than 100 numbers of samples can be detected at a single cycle which falls the lower number of samples tested the lower number of samples because cost is effective.

Result

In diagnostic techniques, the typical fluorescence patterns, observed in liver cells are 27 of homogenous, 23 of speckled (fine & coarse), 7 of rim, 2 of nucleolar & greater of mixed patterns (41) were examined.

On the other hand in HEP₂ cell slides the patterns observed are significant & specificity which help in the diagnosis of disease of specificity disease pattern were observed. In further diagnosis it reveals that the titre value of 1:20 was found to have highly predictive in detecting ANA with those diseases in both substrates. In mouse liver cell the ANA in each disease are 9.3% in SLE, 20% in RA, 20% in JIA, 21.425 in Systemic Sclerosis, 40% in Sjogren's Syndrome, 100% in MCTD, 25% in others. The ANA is successfully detected & identified in the titre value of 1:40 had sensitivity & specificity of 94.3% and 96.07% and greater advantage of liver cells that very small percentage of ANA can be detected by mouse liver tissue & the maintenance of liver tissue is easy, simple & effective method. Certain patterns in mouse liver cannot be detected because the tissue is of animal origin. On the other hand the HEP₂ cell line method when the titre value of 1:80 examine, HEP₂ cells had larger & more impression on the cell nuclei in which the various cells nucleus antibodies can be more easily differentiated by means of fluorescence pattern. In the titre value 1:40 and 1:80 with help in the confirmation of diseases. The prevalence of ANA using HEP₂ 94.8% in SLE, 32% in RA, 4% in JIA, 5% in Systemic Sclerosis, 3% in Sjogren's Syndrome, 1% in MCTD and 2% in other diseases. Finally greater sensitivity & specificity than liver cells because it contains human antigen cells and highly proliferating cells and they even display much higher portion of mitosis SSA, SSB anticentromeric pattern will be predominant. Indirect immunofluorescence ANA test (FANA) provides rapid yet highly sensitive method for ANA detection. Result is reported by three parameters; pattern, titre and type of substrate used. Any pattern of reactivity at a titre of 1:40 and more is taken as positive for autoimmune diseases. For SLE 1:80 is taken as cut off titre. A negative ANA excludes SLE, MCTD and drug induced lupus. Very rarely ANA can be negative in SLE. Mouse liver (animal origin) is taken as the representative of human antigens, but certain antigen like SSA(Ro) and centromere will not be presented. The nuclei will be smaller and appreciation of pattern is difficult when compared HEP₂ cell substrate. ANA positivity for SLE is 92.7% on mouse liver substrate. HEP₂ cell is a human epithelioma (cancer) cell line will have dividing cells with larger nuclei and patterns are better appreciated. ANA positivity 97.4% for SLE (88% for autoimmune disease). Anti-SSA and anti-centromeric patterns will be better appreciated. Mouse liver ANA in house test is inexpensive though animal has to be sacrificed. HEP₂ cell maintenance is expensive procedure are to be taken for preparing in house HEP₂ substrate.

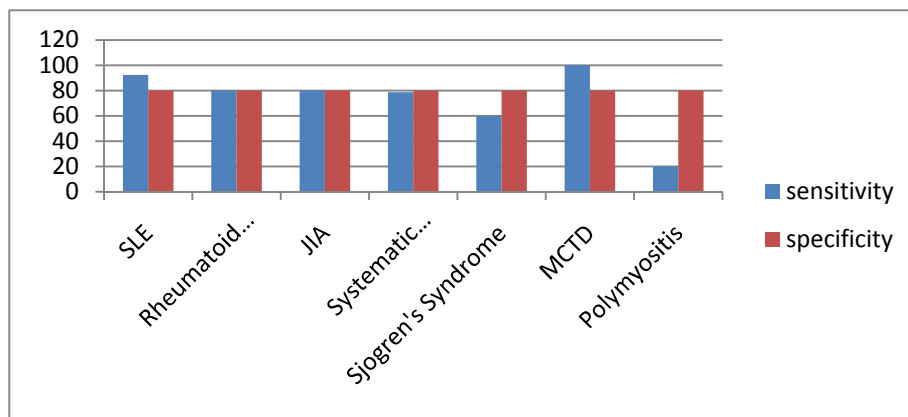
Sensitivity of mouse liver substrate for autoimmune disease is 81.8% and its specificity is 80%. Sensitivity of HEP₂ cell substrate for autoimmune disease is 88.8% and its specificity is 90%. Sensitivity of mouse liver substrate for SLE is 92.3% and its specificity is 80%. Sensitivity of HEP₂ cell substrate for SLE is 97.4% and its specificity is 90%. ANA are the has a chance to miss low titre of antibodies. If ANA when coupled with ELISA both sensitivity and specificity will be improved. After confirmation of ANA, ENA detection can be reported by double immune diffusion

and ELISA. A positive ANA test can be seen in elderly healthy individuals in low titres.

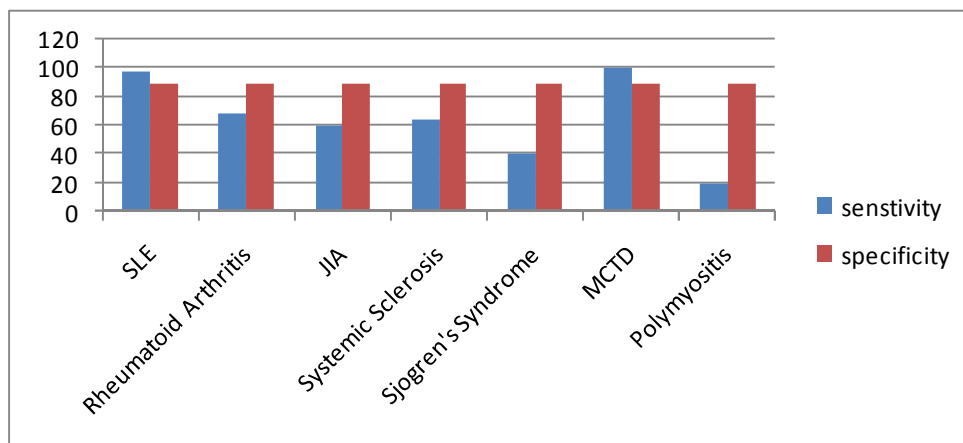
Conclusion

Antinuclear antibodies are the hallmark of autoimmune diseases. They are specific for different diseases not only antibodies involved in disease pathogenesis, but also constitutes the basis for diagnosis and treatment of Connective Tissue Disease.

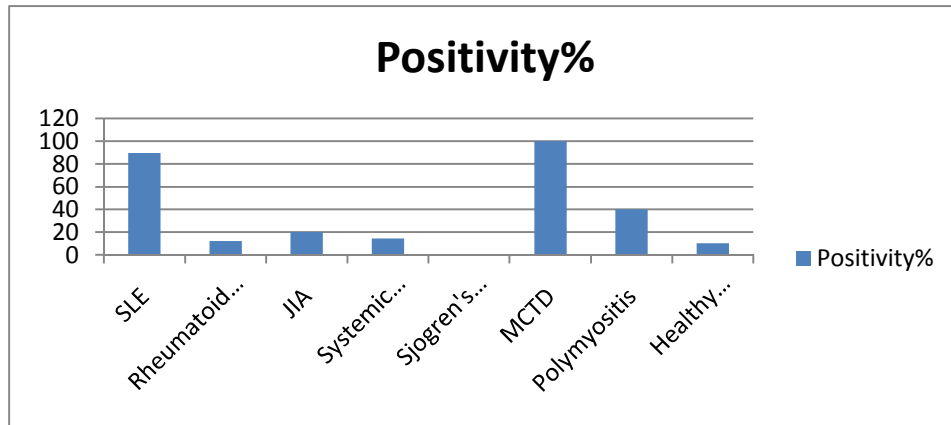
Sensitivity and Specificity on Mouse Liver Substrate



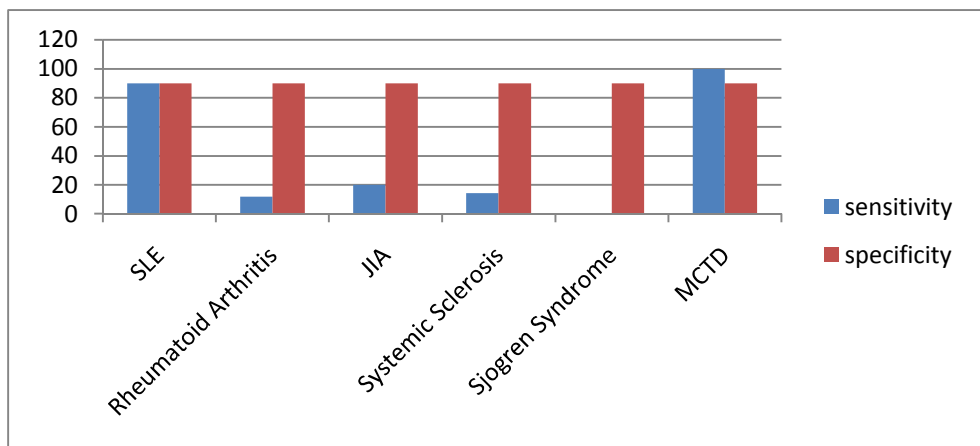
Sensitivity and Specificity on Hep₂ Cell Substrate



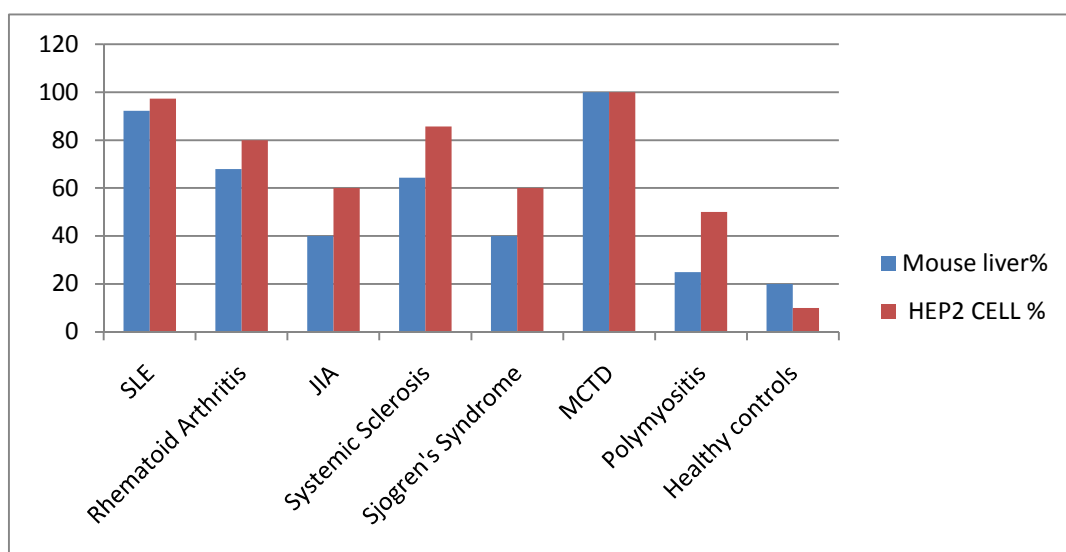
ANA Positivity by ELISA



Sensitivity and Specificity by ELISA



ANA Positivity on Mouse Liver and Hep₂ Cell Substrate



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