



CORRELATION OF INDIRECT IMMUNOFLUORESCENCE PATTERNS ON HEP2000 WITH ANTI DS DNA LEVELS IN PATIENTS WITH SLE.

Dr. Ananthakrishnan Parthasarathy

Assistant Professor of Microbiology, Madras Medical College & Rajiv Gandhi Govt. General Hospital, Chennai

Dr. Meenakshi Subramanian*

Associate Professor of Microbiology, Madras Medical College & Rajiv Gandhi Govt. General Hospital, Chennai *Corresponding Author

ABSTRACT

Background: Antinuclear antibody (ANA) detection is very useful in the diagnosis of systemic lupus erythematosus (SLE). Association of specific autoantibodies with the immunofluorescence pattern of ANA in SLE as noted in Western medical literature has been taken as reference all over the world. However, in developing countries like India such research or data correlating the autoantibodies and their ANA Indirect Immunofluorescence patterns is inadequate.

Objective: To identify the correlation between Indirect immunofluorescence patterns of antinuclear antibody on HEP-2 cell and serum levels of anti- double stranded DNA (anti-dsDNA) in SLE patients.

Material and Methods: Serum samples of 108 SLE patients who were diagnosed by ACR (American College of Rheumatology) classification criteria, attending Rheumatology OPD of a tertiary care hospital during the study period of six months were subjected for ANA testing by Indirect Immunofluorescence (IIF) on HEP-2 cell and anti-dsDNA levels by ELISA.

Results: Out of 108 SLE patients who were all positive by ANA in IIF on HEP-2 cell - exhibited predominantly two fluorescent patterns such as homogeneous in 80 (74%) and speckled 28 (26%) and in those with suspected flare-up homogeneous pattern was strongly associated with anti-dsDNA ($p < 0.05$) and speckled pattern was also predominantly associated with anti-dsDNA ($p < 0.05$).

Conclusion: In SLE patients showing either homogeneous or speckled pattern in ANA IIF with suspected flare-up had elevated anti-dsDNA levels.

KEYWORDS : Antinuclear antibodies (ANA), IIF, dsDNA, ELISA

INTRODUCTION:

Detection of Antinuclear antibodies (ANA) has long been used for the diagnosis and follow-up of systemic autoimmune rheumatic diseases (SARDs). Indirect immunofluorescence assay (IIF) is used in screening and considered to be the gold standard by The American College of Rheumatology to identify the presence of ANA (ANA).⁽¹⁾⁽²⁾ This study intends to correlate the presence of identifiable fluorescence patterns in IIF with the levels of anti-dsDNA determined by ELISA. Antinuclear antibodies (ANA) may be produced against the nucleus, nucleolus, cytoplasm, mitotic apparatus and cell surface antigens and have been broadly classified into nuclear, cytoplasmic and mitotic patterns comprising 30 AC (anti-cell) patterns (AC-0 + 3 divisions) with 2 levels of reporting (expert & competent) according to the International consensus on ANA patterns (ICAP).⁽³⁾

In addition to systemic rheumatic diseases, ANA may also be detected in healthy individuals and also in non-rheumatic diseases (thyroid diseases, infectious diseases, malignant diseases). IF-ANA test results are reported depending on the ANA staining pattern and its titration. ANA staining patterns generally indicate antibodies synthesized against nuclear structures including DNA, proteins and ribonucleoproteins. In a homogeneous pattern-the condensed chromatin of the mitotic cells exhibits solid, uniform fluorescence which is often more pronounced than in the resting cell nuclei and the resting cells exhibit uniform, diffuse fluorescence of the entire nucleus. This characteristic pattern is often the result of anti-dsDNA antibodies.⁽⁴⁾

Positive fluorescence in ANA staining indicates the presence of autoantibodies but does not allow the specific identification of these autoantibodies, the detection of which requires additional techniques such as Enzyme Linked Immunosorbent Assays (ELISA) specific for a particular antibody or Line Immuno Assay (LIA) which detects a number of antigens depending on the antigens coated on each strip are required. Identification of the specific autoantibodies was done by ELISA for the detection of anti-dsDNA antibodies.

MATERIALS AND METHODS:

A total of 108 serum samples of SLE patients referred by rheumatologists in Madras Medical College & Rajiv Gandhi Government General Hospital for ANA testing during the period of July, 2018 to December, 2018 were taken into account in this retrospective study. Blood (5ml) was drawn and serum was separated from the clotted blood samples by centrifugation. Sera were stored at

4°C if testing was planned within 72 hours or at -20°C 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 and ELISA for anti-dsDNA.

Indirect immunofluorescence on HEP-2 cell:

Indirect immunofluorescence was done by a commercially available kit. Serum diluted 1:80 in phosphate buffered saline (SD) was overlaid onto the fixed HEP-2 cells (MEDSOURCE) for 30 minutes at room temperature in a humidified atmosphere to prevent evaporation. Slides were then washed and placed in a Coplin jar with PBS for five minutes and overlaid with fluorescence labeled conjugate, which is antihuman IgG labeled with FITC and incubated for an additional 30 minutes.⁽⁵⁾ After washing again, a drop of mounting medium is placed on the well and a coverslip was placed over the slide, and the slides were read using a fluorescence microscope at 40X.

ELISA:

Antibodies of the IgG class against dsDNA was detected by commercially available ELISA kit (EUROIMMUN, Germany).⁽⁶⁾ Micro-wells were pre-coated with dsDNA antigen coupled with nucleosomes. The calibrators, controls and diluted patient samples (serum) were added to the corresponding wells. Following which the specific 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 and incubated. The conjugate bound to the captured human autoantibody and the excess unbound conjugate was removed by a further wash step. The bound conjugate was visualized by adding TMB/H₂O₂ substrate followed by incubation protected from light which gives a blue reaction product. The intensity of which is proportional to the concentration of autoantibodies in the given sample. 0.5M sulphuric acid was added to each well to stop the reaction. This produces a yellow end point colour, which was read at 450nm within 30minutes of adding the stop solution. The upper limit of the normal range according to the kit is 100 International units.

Homogeneous immunofluorescence staining pattern of double stranded DNA antibodies on HEP-2000 cells show homogeneous nuclear staining in the interphase cells while mitotic cells show uniform staining of the condensed chromatin.

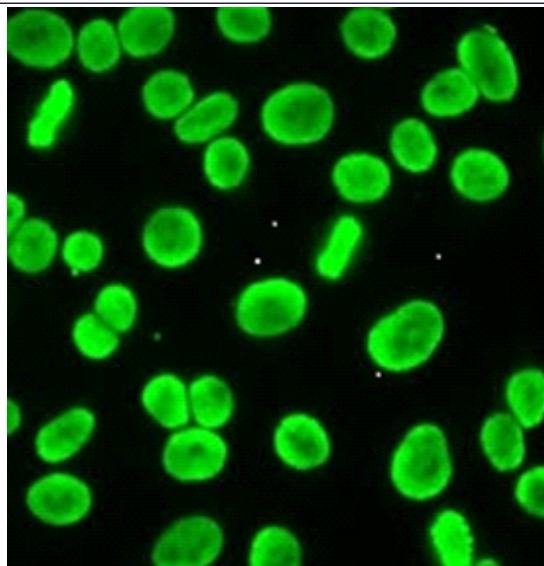


Fig.1 Homogeneous pattern in IIF

Interphase cells show homogeneous nuclear staining while mitotic cells show staining of the condensed chromosome regions.

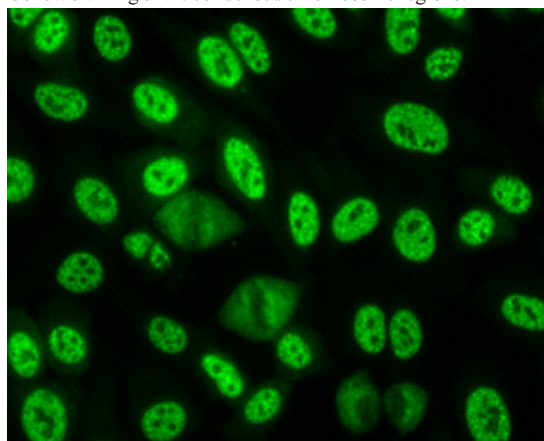


Fig.2 Speckled pattern in IIF

Interphase nuclei: coarse granular positive, mitotic cells: negative

Though ANA IIF test is the recommended method for ANA testing, this method has significant limitations, including that it demands a high degree of technical expertise in performing the test and subjectivity in interpretation of the result.⁽⁷⁾ There are a few indirect immunofluorescence (IIF) nuclear patterns that are commonly reported by most laboratories, namely, homogeneous, speckled, nucleolar, centromere and peripheral/rim. Laboratories performing the ANA IIF test typically report a positive result with a pattern, titer and grading the intensity of fluorescence. This aids the physician when deciding what tests to order next, if any.

Study design: Retrospective observational study

Study period: July 2018 to December 2018

RESULTS:

From the 108 serum samples processed simultaneously for IIF by HEp2000 and anti-dsDNA ELISA, the results were tabulated and analysed. In IIF out of 108 samples processed all of them exhibited interpretable fluorescence patterns. To interpret as negative in IIF, the well must be without fluorescence and a specific pattern. Among the positive IIF patterns 80 were homogeneous and 28 were speckled pattern. The ds DNA ELISA results of the positive IIF samples are as follows. Among the 80 samples positive for homogenous pattern 71 showed increased ds DNA levels in serum and 9 showed ds DNA levels below the cut-off. Out of the 28 samples that showed speckled pattern, only 22 had increased dsDNA levels and 6 had dsDNA levels in the normal range.

Table.1
n=108

Homogenous pattern	Speckled pattern	Total
80	28	108

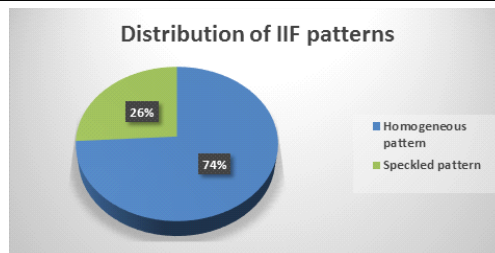


Chart.1

Table.2
n=108

	ds DNA Increased	ds DNA Normal or Decreased
Homogeneous	71	9
Speckled	22	6

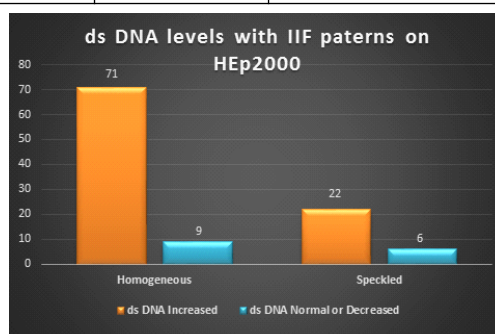


Chart.2

DISCUSSION:

Our study revealed that though ANA IIF patterns though used as an immunological criteria for the diagnosis of SLE – not all patients diagnosed have increased anti dsDNA levels which strongly correlates with disease flare up. While the role of ANA is not known in most of the rheumatic diseases, its role in the pathogenesis of SLE is ascertained and hence has been included in the immunological criteria for SLE diagnosis by the American College of Rheumatology. In SLE, the prototype of autoimmune diseases characterized with multisystemic involvement, ANA, anti-dsDNA and anti-Sm antibodies are included in the diagnostic criteria of the disease.⁽⁸⁾ In patients with SLE, the frequency of anti-dsDNA and anti-Sm was reported by Soto et al.⁽⁹⁾ to be 62% and 35% and by Scholz et al.⁽¹⁰⁾ to be 58% and 20.1%. According to the study done by Sebastian, et al.: ANA and line immunoassay in India, the homogenous pattern was the most common ANA pattern, seen in 46 (45.5%) and the second most commonly occurring ANA pattern in this series was the speckled ($n = 36; 35.6\%$) pattern,⁽¹¹⁾ which is similar to our study.

IIFT is claimed to be affected by subjective interpretation and a lack of standardization, there has been an increasing demand for automated pattern interpretation of immunofluorescence reactions in recent years. Corresponding platforms are already available for evaluation of anti-nuclear antibody (ANA) IIFT on HEp-2 cells, the recommended “gold standard” for ANA screening in the diagnosis of various systemic rheumatic autoimmune diseases.⁽⁷⁾⁽¹²⁾

CONCLUSION:

ANA IIF is a screening (sensitive) and a non-specific test for the diagnosis of SLE which may reveal either a homogeneous or speckled pattern to fit into the ACR criteria for the diagnosis of SLE, the more specific anti-dsDNA antibody levels are for diagnosis (qualitative) and for the monitoring (quantitative) the established cases for disease flares. High levels of anti-dsDNA antibody may precede the development of a clinical flare.

The small percentage of patients with normal anti-dsDNA antibody levels but with or without clinical symptoms of a flare-up probably could be either truly having low anti-dsDNA antibody levels, or the

excess antibodies trapped in the immune complexes and it is decided by the clinical manifestations whether to continue the patient on maintenance therapy or to alter the treatment.

REFERENCES:

1. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO, et al. 2010 Rheumatoid arthritis classification criteria: An American College of Rheumatology/European League Against Rheumatism collaborative initiative. Vol. 62, *Arthritis and Rheumatism*. 2010. p. 2569–81.
2. Gary S. Firestein, Ralph Budd, Sherine E Gabriel, Iain B. McInnes JRO. Kelley and Firestein's Textbook of Rheumatology. 10TH ed. Elsevier Health Sciences, 2016;
3. ICAP Page 1 Letter.pdf.
4. Kale H, Çildağ S, Korkmazgil B, Şentürk T, Kara Y, Akın N. Antinuclear antibodies by IIF-ANA method in systemic rheumatic diseases. *Pamukkale Med J*. 2017;10(3):234–41.
5. The OF. HEp-2000 ® FLUORESCENT ANA-Ro TEST SYSTEM For in vitro Diagnostic Use For Professional Use. 2000; Available from: <https://immunoconcepts.com/wp-content/uploads/2015/12/ANA-FA-2K-En.pdf>
6. Elisa A. Anti-dsDNA-NcX ELISA (IgG) Anti-dsDNA-NcX ELISA (IgG).
7. Bizzaro N, Antico A, Platzgummer S, Tonutti E, Bassetti D, Pesente F, et al. Automated antinuclear immunofluorescence antibody screening: a comparative study of six computer-aided diagnostic systems. *Autoimmun Rev*. 2014 Mar;13(3):292–8.
8. Ardoin SP, Pisetsky DS. Developments in the scientific understanding of lupus. *Arthritis Res Ther*. 2008;10(5):1–8.
9. Perez-Chiney AC, Telich-Tarriba JE, Soto ME, Hernández-Rizo A, Bojalil R, Juárez-Orozco LE, et al. Predictive value of antinuclear antibodies in autoimmune diseases classified by clinical criteria: Analytical study in a specialized health institute, one year follow-up. *Results Immunol* [Internet]. 2013;5:13–22. Available from: <http://dx.doi.org/10.1016/j.rnim.2013.10.003>
10. Scholz J, Grossmann K, Knüttler I, Hiemann R, Sowa M, Röber N, et al. Second generation analysis of antinuclear antibody (ANA) by combination of screening and confirmatory testing. *Clin Chem Lab Med*. 2015;53(12):1991–2002.
11. Kini U, Mullick S, Sebastian W, Roy A. Correlation of antinuclear antibody immunofluorescence patterns with immune profile using line immunoassay in the Indian scenario. *Indian J Pathol Microbiol*. 2010;53(3):433.
12. Gerlach S, Affeldt K, Pototzki L, Krause C, Voigt J, Fraune J, et al. Automated Evaluation of *Crithidia luciliae* Based Indirect Immunofluorescence Tests: A Novel Application of the EUROPattern-Suite Technology. *J Immunol Res*. 2015;2015:1–8.