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COMPARISON OF INDIRECT IMMUNOFLUORESCENCE (IIF) AND ELISA AND THE CORRELATION WITH LINE IMMUNE ASSAY (LIA) IN THE DETECTION OF ANTINUCLEAR ANTIBODIES (ANA) FOR DIAGNOSING AUTOIMMUNE DISORDERS.

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ABSTRACT

Background: Autoimmune disease is a pathological state arising from an abnormal immune response of the body to substances and tissues that are normally present in the body. Antinuclear antibody (ANA) detection by ELISA and indirect immune- fluorescence (IIF) using are commonly used as screening tests. This study was undertaken to compare the screening tests and note their correlation with Line immune assay (LIA). **Aims & Objectives:** To compare IIF and ELISA and confirmation of the positive cases by LIA in detection of Antinuclear antibodies. Material & **Methods:** This retrospective study was conducted in the department of Microbiology, Govind Ballabh Pant Institute of Medical Education and Research (GIPMER), New Delhi. Analysis of data collected over a period of 6 months from October 2022 to February 2023 was done. Serum samples from clinically diagnosed or suspected cases of autoimmune disorders received in the laboratory were processed both by indirect Immuno-fluorescence using hep 2 cells and ELISA. Tests returned positive by either method were subjected to Line Immune Assay (LIA). A total of 602 consecutive samples were received. **Results:** A total of 602 serum samples of the suspected cases of autoimmune disorders were received in the laboratory between October 2022 to February 2023. These samples were tested by both ANA ELISA and ANA IIF. ANA IIF was positive in 148 (24.63 %) at a dilution titre of 1:80 and ANA ELISA was positive in 117 (19.47 %). IIF was found to have a sensitivity of 82.54 % and the sensitivity of ELISA was found to be 46.03 %.IIF had slightly lower specificity of 38.06% compared to ELISA (43.22%), In addition to a comparable positive predictive value, negative predictive value of IIF is more i.e. 84.29% than ELISA 66.34%. **Conclusion:** ANA testing is an important tool in the diagnosis of autoimmune disorders, but has no value in the absence of clinical correlation. A stand-alone positive or negative ANA test offers nothing to the patient or clinician, and hence, an awareness regarding the judicious use of the same is imperative in clinical practice. IIF remains the gold standard test for diagnosing Autoimmune disorders

KEYWORDS : IIF, ANA testing, Autoimmune disorders

INTRODUCTION:-

Autoimmune disease is a pathological state arising from an abnormal immune response of the body to substances and tissues that are normally present in the body. These responses can be systemic or organ specific .Antibodies that specifically react with self-antigens are called autoantibodies and the antibodies that target "normal" proteins within the nucleus of a cell are called antinuclear antibodies (ANA). Small number of autoantibodies can be present in healthy individuals. (1)The presence of a large number of autoantibodies or ANAs can indicate an autoimmune disease. Detection of antinuclear antibodies is the cornerstone in the diagnosis of autoimmune disorders. (2)

Diagnosing an Autoimmune disorder is time consuming and there is invariably a delay due to the overlap of signs and symptoms among the autoimmune diseases. This also delays the appropriate treatment. For timely diagnosis, selection of appropriate screening test with higher sensitivity is of utmost importance. (3)

Antinuclear antibody (ANA) detection by ELISA and indirect immune- fluorescence (IIF) using Hep 2 Cells (human laryngeal epidermoid carcinoma cell line type 2) are commonly used as screening tests. Indirect immune fluorescence technique (ANA-IIF) is a valuable screening tool for autoimmune connective tissue diseases (CTDs), though it is non-specific. The test is positive in many autoimmune conditions such as SLE, autoimmune hepatitis, primary biliary cirrhosis. (4)

For the last many years ANA testing with ELISA technique has been introduced aiming to save time and efforts needed for ANA-IIF, but ELISA still continues to have a lower sensitivity compared to IIF. Moreover, fluorescence pattern in IIF predicts the presence of certain specific antibodies in serum. Auto-immune antibodies are disease specific and detection of these specific antibodies aids the diagnosis and treatment of certain autoimmune disorders. This also helps in reducing the sample demand for the line immunoassay (LIA) which is a more expensive test. (5,6)

IIF by using HEP-2 cells as the substrate allows the detection of more than 50 auto-antibodies against more than 30 different nuclear and cytoplasmic antigens. These antibodies are not only involved in the disease pathogenesis but also constitute the basis for diagnosis and treatment of connective tissue disorder. Their detection with high sensitivity and specificity is therefore of utmost importance. (7)

This study was undertaken to compare the screening tests and note their corelation with Line immune assay (LIA).

OBJECTIVES:

- To compare IIF and ELISA and confirmation of the positive cases by LIA in detection of Antinuclear antibodies.

Inclusion Criteria:
All clinically suspected cases of autoimmune disorders for which sample requests for ANA was sent were accepted in the microbiology laboratory.

Exclusion Criteria: Inappropriate samples, haemolysed and lipemic samples were excluded

MATERIALS AND METHODS:

This retrospective study was conducted in the department of Microbiology, Govind Ballabh Pant Institute of Medical Education and Research (GIPMER), New Delhi. Analysis of data collected over a period of 6 months from October 2022 to February 2023 was done.

Serum samples from clinically diagnosed or suspected cases of autoimmune disorders received in the laboratory were processed both by indirect Immuno-fluorescence using hep 2 cells and ELISA. Tests returned positive by either method were subjected to Line Immune Assay (LIA).

A total of 602 consecutive samples were received (duplicate samples were excluded). A single sample from each patient was tested using ELISA (ANA screen 8 IgG ELISA SBA diagnostics) and IIF (HEP 2000, Immunoconcepts, USA), following the manufacturer's instruction. Serum for IIF was tested at a screening dilution of 1:80. Diluted sera were placed on ANA wells provided in kit which allowed anti-nuclear antibodies present in sera to bind with the corresponding antigens present on the slides. The slides were then incubated for 30 mins and were then rinsed with phosphate buffer. Fluorescein labelled antihuman globulin provided in test kit was added, incubated for 30 more mins, rinsed, followed by mounting with mounting medium and slides were examined under the fluorescent microscope for staining pattern and intensity. When observed under IF microscope varied patterns with different intensity was seen.IIF was independently read by two senior residents. Discrepant results were verified by the professor in-charge.

ANA ELISA was performed on same serum samples. Sera was diluted and added to the nuclear antigen coated wells provided by the kit manufacturer. Wells were rinsed to provide only bound ANA on the wells. Enzyme conjugate was added to these wells with antigen antibody complexes. Wells were again rinsed to remove excess conjugate and incubated. Intensity of colour was then read on ELISA reader.

The results thus obtained by both IIF and ELISA were then compared. Sera which were positive, by either of the procedures were, subjected to Line Immunoassay (LIA) for specific antibodies. The detection of specific antibodies was considered in the final results.

IMTEC-ANA-LIA XL Profile facilitates multiplex detection of human IgG antibodies. The assay measures antibody binding to 17 antinuclear antigens: dsDNA, nucleosomes, histones, Smith (Sm)D1, U1-small nuclear RNP (UI-snRNP), ribosomal p protein (RPP)/P0, proliferating cell nuclear antigen (PCNA), SS-A/Ro52, SS-A/Ro60, SS-B/La, centromere protein (CENP)-B, Scl70, Jo-1, PM-Scl, antimitochondrial antibody (AMA)-M2, Mi-2, and Ku, DFS 70. These antigens are marked on a strip with three positive control lines (Reference line, functional control, cut off control). Result is read by checking the Intensity of bands using a scanner. The intensity value of the cut off control line was used as the positive cut-off value. The results of each antigen band were interpreted as negative or positive.

Institutional Ethics Committee Clearance: Approval was sought from the institutional Ethics committee before the start of the study. F.1/IEC/MAMC/94/06/2022/No 27 dated 02.02.2023.

Statistical Analysis: The data was entered in MS EXCEL spreadsheet and analysis was carried out using SPSS software 22.0 version. P-value of less than 0.05 was considered statistically significant. Appropriate use of charts, tables, diagrams was undertaken wherever required.

RESULTS:-

A total of 602 serum samples of the suspected cases of autoimmune disorders were received in the laboratory between October 2022 to February 2023. These samples were tested by both ANA ELISA and ANA IIF.

ANA IIF was positive in 148 (24.63 %) at a dilution titre of 1:80 and ANA ELISA was positive in 117 (19.47 %). More details are mentioned in Table 1.

Table 1:- ELISA and IIF distribution.

ELISA and IIF	Frequency	Percentage
ELISA positive	117	19.43%
IIF positive	148	24.63%
IIF positive and ELISA negative	67	11.12%
IIF positive and ELISA positive	81	13.45%
IIF negative and ELISA positive	36	5.9%

Table 2:- LIA distribution.

LIA	Frequency	Percentage
Negative	155	71.10%
Positive	63	28.90%
Total	218	100.00%

Total 218 samples were subjected to LIA, these included, cumulative positive samples (148 + 36=184), determined positive by ELISA and IIF and 34 negative samples, were then subjected to LIA. 63 (28.90 %) of these samples tested positive on LIA. These subsets of samples were true positives as LIA is a confirmatory test. However, the 155 samples which tested negative on LIA, included 34 true negatives and remaining 121 samples can't be deemed as true negatives because LIA only tests for antibodies against 17 antigens present on LIA strips. Antigens not detected by LIA may be detected by ELISA or IIF.

Table 3:- Inter-rater kappa agreement between ELISA and LIA.

ELISA	LIA		Total	P value	Kappa
	Negative(n=155)	Positive(n=63)			
Negative	67 (30.73%)	34 (15.60%)	101 (46.33%)	0.149	-0.086
Positive	88 (40.37%)	29 (13.30%)	117 (53.67%)		
Total	155 (71.10%)	63 (28.90%)	218 (100.00%)		

Table 4:-Inter-rater kappa agreement between IFA and LIA.

IIF	LIA		Total	P value	Kappa
	Negative(n=155)	Positive(n=63)			
Negative	59 (27.06%)	11 (5.05%)	70 (32.11%)	0.003	0.147
Positive	96 (44.04%)	52 (23.85%)	148 (67.89%)		
Total	155 (71.10%)	63 (28.90%)	218 (100.00%)		

When evaluating the positive results of either test and comparing the results with LIA, inter rater kappa agreement between IIF and LIA is statistically significant with p value of 0.003.

Table 5:- Sensitivity, specificity, positive predictive value and negative predictive value of IFA and ELISA for predicting positive LIA.

Variables	ELISA	IIF
Sensitivity (95% CI)	46.03% (33.39% to 59.06%)	82.54% (70.90% to 90.95%)
Specificity (95% CI)	43.23% (35.30% to 51.41%)	38.06 % (30.39 % TO 46.20 %)
AUC (95% CI)	0.45(0.38 to 0.51)	0.6(0.53 to 0.67)
Positive Predictive Value (95% CI)	24.79% (17.27% to 33.62%)	35.14% (27.48% to 43.40%)
Negative Predictive Value (95% CI)	66.34% (56.25% to 75.44%)	84.29% (73.62% to 91.89%)
Diagnostic accuracy	44.04%	50.92%

IIF was found to have a sensitivity of 82.54 % and the sensitivity of ELISA was found to be 46.03 %.IIF had slightly lower specificity of 38.06% compared to ELISA (43.22%), In addition to a comparable positive predictive value, negative predictive value of IIF is more i.e. 84.29% than ELISA 66.34%.

DISCUSSION:

Due to the high prevalence of autoimmune disorders, there is an urgent need for a reliable screening test for the same. ANA testing by immunofluorescence has been used for more than 40 years as a screening method for autoimmunity and is still the standard method. Several different types of alternative assays based on ELISA or multiplex beads assay have been developed in an attempt to replace immunofluorescent ANA, however due to the advantages of ANA testing by IIF including its higher sensitivity, large number of autoantibodies that can be detected using the HEp-2 cells and the pattern of staining can provide a clue to the diagnosis, make ANA IIF still the method of choice and a preferable method used for screening.

In our study, amongst the 602samples tested, ANA by IIF showed positivity in 148 (24.63%) cases while ANA by ELISA showed positivity in 117 (19.4%) cases. ANA positivity by IIF was higher as compared with ELISA. The sensitivity of IIF was found to be 82.54 % and the sensitivity of ELISA was 46.03 %. This higher sensitivity makes indirect IIF technique as the method of choice for the detection

of ANA. This finding is in concordance with findings of Tayde, et al. (4) Solid phase assays have low sensitivity, hence less suitable for screening. Sensitivity varies as per the test kit from 69% to 98% with an average of 87%. (5) Many antigens present in HEp-2 cells are absent in ELISA, hence, false-negative results may be high in ELISA assays.
IIF had slightly lower specificity of38.06% compared to ELISA (43.22%) but had a comparable positive predictive value. Negative predictive value of IIF was higher than ELISA (84.29% vs 66.34% respectively).

81 samples (13.45 %) were positive by both the methods. Most samples that were positive by ELISA, were also positive by IIF method. This finding was consistent with finding of Copple, et al. They also found results were mostly similar when they compared different ELISA and IIF methods. (7)

The results suggest that both ELISA and IIF are good as screening tests but IIF being highly sensitive is preferred over ELISA. Moreover, IIF has an added advantage as it gives us information about the ANA IIF pattern, staining patterns may provide a clue to the underlying CTD, as certain ANA patterns are associated with the presence of autoantibodies to certain nuclear antigens which in turn are associated with certain clinical state like autoimmune hepatitis, SLE, CREST syndrome, Sjogren's syndrome, scleroderma, mixed connective disorders. (6)

Different ANA patterns are associated with one or other Connective Tissue Disorders. A systematic approach has to be followed while performing these tests. Therefore, after initial screening, further tests for specific autoantibodies are to be done, based on the clinical features, possible diagnosis. and IIF-ANA staining patterns. (5)

Qin, et al in 2009 undertook a study comparing HEp-2 IIF testing with ELISA for ANA testing. They concluded that combining two tests for screening enhances the accuracy of the results. (9)

ANA-LIA was complementary with the results of ANA IIF and ANA ELISA as LIA measures antibody binding to 17 antinuclear antigens only .When evaluating the positive results of either of the tests and comparing the results with LIA, inter rater kappa agreement between IIF and LIA was statistically significant with p value of 0.003, suggesting that IIF is a better test to be used and if specific tests are not available ,IIF can be used for aiding the clinician in diagnosing when clinical history is suggestive of the autoimmune disorder.

A positive ANA test gives useful information about the onset of a disease many years prior and is useful to know about the prognosis, in terms of the complications and clinical course. Hence more awareness on ANA testing is the need of the hour and a reliable screening test needs to be adopted. (8)

ELISA has its own advantages of having better reproducibility, being less labour-intensive and less time-consuming, and not requiring training and expertise unlike IIF. These factors ensure reliability and consistency. The major drawbacks of ELISA are the use of a limited number of purified or recombinant auto-antigens, lack of standardization and the prevalence of "false negative" results. These limitations of ELISA make IIF a better screening test. (10)

A positive ANA test is a part of the classification criteria of most Connective Tissue Disorders. A positive ANA is a fundamental parameter for diagnosis of autoimmune hepatitis (AIH) as an organ-specific autoimmune disease (8)

The positive rate of the ANA-IIF test in subjects with suspecting autoimmune diseases was 24.63 %. The sensitivity and simplicity of an ANA test makes it extremely popular initial test to evaluate for lupus in particular. Since most people (more than 95% of individuals) with lupus will test positive, a negative ANA test can be helpful in excluding that diagnosis. The prevalence of ANAs in healthy individuals is about 3 - 15% (11). However, a positive ANA reading alone does not indicate an autoimmune disease. It is important to have a clinical history suggestive of autoimmue disorder.

36 samples were IIF negative but positive by ELISA, these were the samples processed early during the start of IIF in our department. The possible reason for the same was failure to interpret positive patterns early on during the course of study as IIF had just been introduced in our institution. Other reasons for sub-optimal interpretation could be a lack of standardization techniques when IIF was introduced in our institute. So ideally for institutes which are in the process of introducing IIF, should continue reporting by both the methods till the method gets standardized.

Although new assays can be cost efficient and are somewhat comparable to immunofluorescent ANA, but ANA by IIF is still the gold standard for the screening of ANA, it has been used for over 40 years as a first-step screening test for autoimmune diseases and is still continues to be the same. (12)

CONCLUSION: -

ANA testing is an important tool in the diagnosis of autoimmune disorders, but has no value in the absence of clinical correlation. A stand-alone positive or negative ANA test offers nothing to the patient or clinician, and hence, an awareness regarding the judicious use of the same is imperative in clinical practice. IIF remains the gold standard test for diagnosing Autoimmune disorders.

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