

## Evaluation Of A Semi-Automated Indirect Immunofluorescence Assay Using Validated In-House Hep-2 Cell Slides As A Reference Standard For Antinuclear Antibody Detection

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### ABSTRACT

**Objective:** To evaluate the analytical performance of a semi-automated assay for detection of antinuclear antibodies by indirect immunofluorescence.

**Study Design:** Cross-sectional study

**Place and Duration of Study:** Department of Immunology, Armed Forces Institute of Pathology (AFIP), Rawalpindi, Pakistan, from Jan to Jun 2023

**Methodology:** A total of thirty-six serum samples were selected from patients with antinuclear antibody-positive systemic autoimmune rheumatic diseases (n = 26) and from healthy controls (n = 10). Samples were applied on in-house HEp-2 cell slides and then subsequently stained and interpreted by two experienced observers independently. In parallel, ANA tests were also performed using a semi-automated indirect immunofluorescence assay (IFA) benchtop analyzer (Akiron NEO) and software using a standard kit as per the manufacturer's instructions. Positive/negative results, staining patterns, and intensities were compared between in-house HEp-2 cell slides as a reference standard and the semi-automated assay. Relative sensitivities, specificities, and degree of concordance were assessed by overall percentage agreement or by Cohen's k.

**Results:** Among a total of 36 samples, in-house HEp-2 cell slides yielded 26 (72.22%) positive and 10 (27.78%) negative. The semi-automated IFA system yielded 21(58.33%) positive samples and 15(41.66%) negative samples. Overall relative sensitivity, specificity, and concordance of the semi-automated IFA system were 65.38%, 60%, and 63.88% (k = 0.21), respectively (p = 0.26).

**Conclusions:** The semi-automated IFA system (Akiron NEO) has fair agreement with in-house HEp-2 cell slides with respect to positivity/negativity and pattern recognition but poor agreement for intensity. This makes semi-automated evaluation of ANA relatively less suitable for routine testing.

**Keywords:** Antinuclear Antibodies, HEp-2 Cells, Indirect Immunofluorescence, Semi-automated Indirect Immunofluorescence Assay

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### INTRODUCTION

Antinuclear antibodies (ANA) are invaluable biomarkers of autoimmune disorders. ANA detection by indirect immunofluorescence assay (IFA) on human epithelial type-2 cells (HEp-2 cells) is considered the “gold standard” for screening of systemic autoimmune rheumatic diseases (SARDs).<sup>1,2</sup> The American College of Rheumatology also recommends ANA detection by indirect immunofluorescence using HEp-2 cells for diagnostic and prognostic purposes in cases of SARDs.<sup>3</sup> There is a surge of autoimmune disorders in the last decade, which may be multifactorial, involving genetic predisposition, environmental triggers, hormonal collaboration, chronic infections, aging, and drugs.<sup>4</sup>

With increasing prevalence of autoimmune disorders, requests for ANA testing have increased to exclude autoimmunity.<sup>5</sup>

ANA detection on HEp-2 cell slides by IFA is the most sensitive method of screening. The HEp-2 IFA test not only detects the presence or absence of autoantibody but also provides information about staining patterns (including nuclear, cytoplasmic and mitotic patterns) and titres.<sup>6</sup> Lack of standardization, the labor-intensive nature of the procedure for IFA, and investigator abilities can result in intra- and inter-laboratory differences.<sup>7</sup> To overcome the hurdles of standardization, objectivity, labor-intensive work, and high throughput of results, many strategies have been designed as a substitute to IFA, including enzyme-linked immunosorbent assay (ELISA), immunoblots, and multiplex bead assays.<sup>8</sup> But these recent techniques showed variable results, which may be due

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to differences in procedure, source of antigen extraction, concentration, and changes in antigen binding sites during processing.<sup>9</sup> Based on these issues with new assays, the American College of Rheumatology (ACR) recommends IFA as the “gold standard” for ANA screening.<sup>3</sup> For the standardization of ANA results by IFA, the International Consensus on ANA Patterns (ICAP) has been developed with the motive to reach an agreement for categorization of different fluorescence patterns and implement guidelines for reporting and interpreting 29 distinct patterns.<sup>10</sup>

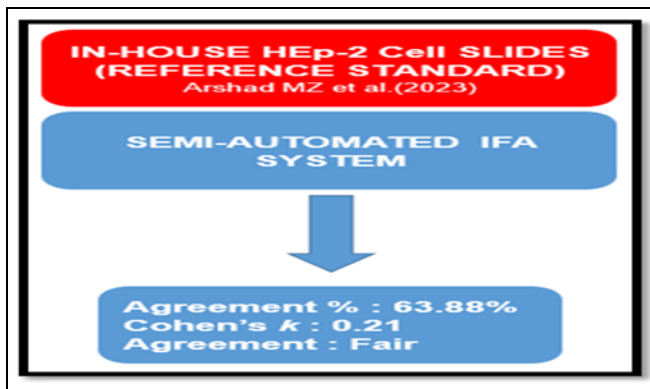


Figure: Analytical Evaluation of Semi-Automated Assay (n = 36)

With ever increase demand for ANA testing and standardization by ICAP guidelines, different automation machines are now available in the market for IFA, in which no intervention is needed in slide preparation and titration. These automated machines are attached to an autofocus fluorescence microscope, which provides imaging and interpretation of results. The ANA automation is an area of extensive clinical research having application of paramount importance. To the best of our knowledge, to date in Pakistan, no previous study has been conducted to find out and document the analytical performance of a semi-automated assay for detection of antinuclear antibodies. The present study aimed to evaluate the analytical performance of a semi-automated ANA IFA analyzer(Akiron Neo) in a real-life laboratory scenario by keeping in-house HEP-2 cell slides as a reference standard so that these methods may be used in the future depending upon the present results.

## METHODOLOGY

This cross-sectional (concordance) study was carried out at the Department of Immunology, Armed Forces Institute of Pathology, Rawalpindi, Punjab, Pakistan, from Jan to Jun 2023. The study involved

non-probability sampling, and the sample size (n) was calculated using sensitivity specificity arifin sample size calculator(online) by keeping the following assumptions: prevalence(p) of ANA as 9%, sensitivity and specificity of indirect immunofluorescence 100%, specificity 93%, precision(acceptable) 0.10, and confidence level as 95 %. Calculated sample size was 28; to cater to the design effect, a final sample size of 36 was selected.<sup>11</sup>

**Inclusion Criteria:** Patients of either gender with ages ranging from 1 month to 85 years presenting with antinuclear antibody positivity with common patterns including homogeneous, peripheral, coarse speckled, fine speckled, centromere, nucleolar, and ANA-negative healthy controls.

**Exclusion Criteria:** Patients with hemolyzed, lipaemic, or icteric serum samples were excluded from the study.

The study was formally approved by the institutional ethics committee (IRB/22/953), and written informed consent from patients and controls was obtained.

A total of 36 serum samples were selected, including ANA-positive (n = 26) and ANA-negative samples from healthy controls (n = 10). Samples were applied at a 1:40 dilution on in-house HEP-2 cell slides, which were used as a reference standard<sup>15</sup>, then subsequently stained with FITC-labelled anti-human IgG antibodies. Two experienced observers interpreted in-house HEP-2 cell slides independently without knowledge of the interpretation by the other observer. If two observers disagreed, a consensus was reached by mutual discussion.

In parallel, ANA tests were also performed using a semi-automated indirect immunofluorescence assay(IFA) benchtop analyzer(Akiron NEO) and software, using the Cytobead ANA standard kit as per instructions from the manufacturer. Samples were applied at a 1:80 dilution, and then slides were stained with FITC-labelled anti-human IgG antibodies. Slides were then loaded into the semi-automated IFA system, which takes digital snaps and store on the computer system. The positivity/negativity, pattern, and intensity of ANA were analyzed using software which interpret the pattern of the stored images using a saved algorithm and also automatically predicts intensity.

For quality control, two controls (positive and negative) were tested in parallel on in-house HEP-2

cell slides and cytobead ANA slides. Fluorescence on in-house HEp-2 cell slides was observed using an immunofluorescent microscope BA-310, and observations were noted in a predesigned proforma. Qualitative variables (gender, positivity/pattern, intensity) were expressed as frequencies and percentages, and quantitative variables (age) were expressed as mean  $\pm$  SD. Data were analyzed using Microsoft Excel version 13.0. Visual reading by both independent observers on in-house HEp-2 cell slides was used as a reference standard, and positive/negative results, staining patterns, and intensities were compared between in-house HEp-2 cell slides and the semi-automated IFA system; overall percentage agreement and Cohen's  $k$  was used to determine the degree of concordance. Interpretation of Cohen's  $k$  values was as follows: poor  $\leq 0.20$ , fair 0.21–0.40, moderate 0.41–0.60, good 0.61–0.80 and very good agreement as 0.81–1.00. The  $p$  values  $\leq 0.05$  were considered statistically significant and calculated using Fisher's exact test.

## RESULTS

Among the total 36 samples, in-house HEp-2 cell slides yielded 26(72.22%) positive and 10(27.78%) negative, and the semi-automated IFA system yielded 21(58.33%) positive samples and 15(41.66%) negative samples. Using visual reading on in-house HEp-2 cell slides as the reference standard, overall sensitivity, specificity, and concordance of the semi-automated IFA system were 65.38%, 60%, and 63.88% ( $k = 0.21$ ), respectively ( $p = 0.26$ ). For ANA positivity by both in-house HEp-2 cell slides and the semi-automated IFA system, the overall percentage agreement came out to be 37.68 %. For ANA negativity by both in-house HEp-2 cell slides and the semi-automated IFA system, the overall percentage agreement was calculated to be 38.46%. Overall percentage agreement for ANA intensity by both in-house HEp-2 cell slides and the semi-automated IFA system was 41.26%. For pattern recognition by both in-house HEp-2 cell slides and the semi-automated IFA system, the overall percentage agreement was calculated as 37.68%. Inter-observer agreement between two independent observers for ANA positivity, intensity, and pattern determination on in-house HEp-2 cell slides was 100 %. The analytical performance of the semi-automated IFA system for differentiating between positive and negative ANA results is summarized in Table-I.

**Table-I: Analytical Performance of Semi-Automated IFA By Keeping Hep-2 Cell Slides As Reference Standard (n = 36)**

|                           | In-House HEp-2 Cell Slides |           |
|---------------------------|----------------------------|-----------|
|                           | Positive                   | Negative  |
| Semi-Automated IFA System |                            |           |
| Positive                  | 17(47.22%)                 | 4(11.11%) |
| Negative                  | 9(25.00%)                  | 6(16.66%) |

Sensitivity = True Positive / (True Positive + False Negative)  $\times 100 = 65.38\%$

Specificity = True Negative / (True Negative + False Positive)  $\times 100 = 60\%$

Positive Predictive Value = True Positive / (True Positive + False Positive)  $\times 100 = 80.95\%$

Negative Predictive Value = True Negative / (True Negative + False Negative)  $\times 100 = 40\%$

Diagnostic Accuracy / Overall percentage agreement = (True Positive + True

Negative) / All Subjects  $\times 100 = 63.88\%$

Overall agreement (oa) = (True Positive + True Negative) / All Subjects = 0.63

Probability of chance agreement (ca) = [(TP + FP)(TP + FN) + (FN + TN)(FP + TN)] / (TP + FP + FN + TN) $^2 = 0.53$

Overall Concordance (Cohen's  $k$ ) = (oa - ca) / (1 - ca) = 0.21

Fisher's exact test value ( $p$  value) = 0.26

**Table-II: Concordance for Semi-Automated IFA And In-House Hep-2 Cell Slides As Reference Standard (n = 36)**

| Immunofluorescence | Parameters    |             |           |
|--------------------|---------------|-------------|-----------|
|                    | Agreement (%) | Cohen's $k$ | Agreement |
| Positivity         | 37.68 %       | 0.24        | Fair      |
| Negativity         | 38.46%        | 0.21        | Fair      |
| Intensity          | 41.26%        | 0.11        | Poor      |
| Pattern            | 37.68%        | 0.23        | Fair      |

## DISCUSSION

The present study is the most extensive Pakistan-based single-center study assessing the analytical performance of a semi-automated analyzer (Akiron NEO) used for ANA testing in a real-life laboratory scenario. The semi-automated IFA system demonstrated fair agreement with visual interpretation of in-house HEp-2 cell slides for ANA positivity and negativity. Fair agreement was also observed for recognition of ANA staining patterns.

However, agreement for fluorescence intensity was poor between the semi-automated system and visual interpretation. These findings indicate limited suitability of the current semi-automated approach for routine ANA evaluation.

Tipu *et al.*, demonstrated that Helios has good concordance in differentiating between positive and negative results. However, it has certain limitations in positive/negative differentiation, pattern recognition, and titer determination, necessitating further verification of results by visual reading.<sup>12</sup> In the present study, relative sensitivity, specificity, and concordance of the semi-automated IFA system using visual reading, with in-house HEp-2 cell slides as the reference standard, were 65.38, 60, and 63.88% ( $k = 0.21$ ), respectively ( $p = 0.26$ ), which deviated considerably from results of previous studies like Arshad *et al.*, demonstrating use of automated analyzers.<sup>13</sup>

Van et al., conducted study in scleroderma patients and as per their findings by using kappa coefficient, there was good concordance between multiplex bead technology (MULTIPLEX-ANA) and the presence of Scl-70, RNP and centromere antibodies (0.76; 95% CI 0.59, 0.92), but there was no concordance between MULTIPLEX-ANA and other IIF-ANA patterns, including the nucleolar pattern (-0.40; 95% CI -0.64, -0.16).<sup>14</sup> Although there is comparable performance for ANA IIF using automated and conventional visual reading for differentiation of positivity/negativity, discrepancies among patterns have been noted. Few automated IIF systems may misinterpret results when antibodies interact with few and specific cellular components (Golgi apparatus, nuclear membrane, or nuclear dots). Such misinterpretation may have severe implications in clinical scenarios, thus stressing the importance of visual verification.<sup>15</sup>

Sowa *et al.*, described that autoAb screening and confirmatory testing can be combined for the first time. They also discussed the history of autoAb assay techniques in this context and gives an overview and outlook of the recent progress in emerging technologies.<sup>17</sup>

Computer-assisted diagnosis (CAD) systems for ANA IIF on HEp-2 cells improve result reproducibility, harmonization, and objective fluorescence quantification. Proper calibration and automated image interpretation are essential for standardized and reliable results. By facilitating quality-control procedures, CAD systems may further support laboratory quality assurance and certification.<sup>17</sup>

A study conducted by Ricchiuti *et al.*, showed that the concordance between EURO Pattern software and the observer's visual reading regarding discrimination of positive and negative samples was higher (95.6%) as compared to the present method (89.4%). Furthermore, the software showed agreement between the observer's visual reading and the software in 80.6-97.5% of patterns and 71.0-93.8% of titers.<sup>18</sup>

#### LIMITATION OF STUDY

This study had several limitations. Its pilot design included a small sample and only common ANA patterns—homogeneous, peripheral, coarse speckled, fine speckled, centromere, and nucleolar—limiting assessment of the semi-automated IFA to these patterns. Extractable nuclear antigen and disease status were not considered because the study focused on agreement between automated ANA results and

visual HEp-2 slide assessments under routine conditions. Single-center design may also introduce inter-observer bias, warranting multicenter studies; however, two independent observers achieved 100% agreement. Future studies should evaluate these factors.

#### CONCLUSION

In conclusion, ANA results reported by the semi-automated IFA system have fair agreement with visual interpretation of the results on in-house HEp-2 cell slides with respect to negativity/positivity and pattern recognition, but poor agreement for intensity. This makes semi-automated evaluation of ANA relatively less suitable for the routine setting. Upgrading, optimizing analyzers, and developing software with better reliability and diverse capabilities may allow semi-automated ANA IFA analyzers to be fully integrated into the increasing workload of clinical laboratories.

**Conflict of Interest:** None.

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#### Authors' Contribution

Following authors have made substantial contributions to the manuscript as under:

MZA & MOR: Data acquisition, data analysis, critical review, approval of the final version to be published.

MH & DA: Study design, data interpretation, drafting the manuscript, critical review, approval of the final version to be published.

AT & MAH: Conception, data acquisition, drafting the manuscript, approval of the final version to be published.

Authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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