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Evaluation of In-House Rat Liver Substrate Slides by Keeping Validated In-House Hep-2 Cell Slides as Reference Standard - An Experience from Tertiary Care Centre

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ABSTRACT

Objective: To evaluate analytical performance of in-house rat liver substrate slides for detection of antinuclear antibodies by indirect immunofluorescence.

Study Design: Cross-sectional(concordance) study.

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

Methodology: Total thirty-six serum samples were selected from patients with antinuclear antibodies positive systemic autoimmune rheumatic diseases (n = 26) and from healthy controls (n = 10). Sample were applied on in-house rat liver substrate slides and in-house HEp-2 cell slides in parallel, then both slides were subsequently stained and interpreted by two experienced observers independently. Positive/negative results, staining patterns and intensities were compared between in-house HEp-2 cell slides as a reference standard and in-house rat liver substrate slides. Relative sensitivities, specificities, 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. Subsequently in-house rat liver substrate slides yielded 27(75.00%) positive and 9(25.00%) as negative. Overall relative sensitivity, specificity and concordance of in-house rat liver substrate slides and in-house HEp-2 cell slides was 100.00%, 90.00% and 97.22% (k = 0.92) respectively ($s < 0.001$).

Conclusions: In-house rat liver substrate slides showed good agreement with in-house HEp-2 cell slides in distinguishing ANA positivity/negativity and intensity but have limitation in pattern recognition. They can be used as valuable and cost effective substrate for ANA screening in resource limited conditions especially at peripheral setups.

Keywords: Antibodies, Antinuclear, Fluorescent Antibody Technique, HEp-2 Cells, Indirect Immunofluorescence, Rat, Liver Substrate Slides

How to Cite This Article: *Arshad MZ, Riaz MO, Hussain M, Ahmad D, Tanveer A, Alam M. Evaluation of In-House Rat Liver Substrate Slides by Keeping Validated In-House Hep-2 Cell Slides as Reference Standard - An Experience from Tertiary Care Centre. Pak Armed Forces Med J 2026; 76(Suppl-7): S1057-S1061. DOI: https://doi.org/10.51253/pafmj.v76iSUPPL-7.10691*

INTRODUCTION

Systemic autoimmune rheumatic diseases (SARDs) are heterogeneous group of autoimmune disorders showing varied clinical presentation, disease course, and treatment response.[1] Among SARDs, rheumatoid arthritis, sjogren's syndrome, systemic lupus erythematosus and mixed connective tissue diseases are most debilitating diseases sharing major portion of morbidity and mortality.[2] Genetic and environmental factors are major risk factors, among later smoking, ultraviolet light, diet, and microbiota are most important.[3]

Autoantibodies targeting intracellular antigens mainly in the nucleus, called as antinuclear antibodies (ANA), are serological hallmark and immunodiagnostic tool of SARDs, having pivotal role in the diagnosis/classification. First described more than 50 years ago ANA remains screening tool for majority of autoimmune rheumatic diseases and also part of EULAR/ACR criteria of few disorders.[4-6] However, it is yet to clear whether ANA are bystanders or pathogenic.7 Autoantibody Standardization Committee (ASC) established International Consensus on ANA Patterns (ICAP) in 2014 during the Workshop held in Brazil, with the goal to promote harmony and understanding of HEp-2 cell indirect immunofluorescence assay(IFA) staining patterns, nomenclature as well as use in clinical scenario.[8-9]

ANA testing involves two steps. First, screening to detect the ANA irrespective of the antigen specificity. Second, for positive screening assay to characterize the antigen specificity. Methods available in clinical laboratories for screening ANA include

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Received: 31 Jul 2023; revision received: 14 Feb 2024; accepted: 19 Feb 2024

indirect immunofluorescence assay (IFA), enzyme linked immunosorbent assay (ELISA) and multiplex immunoassay (MIA). Indirect immunofluorescence (IIF) detects antibodies that bind to tissue substrates, which for ANAs, is mostly fixed human epithelial type-2 cells(HEp-2) or rat liver substrate.[10]

As per our knowledge, no previous local study has been conducted so far to find out and document analytical performance of in-house rat liver substrate slides for detection of antinuclear antibodies. The goal of present study was to evaluate the analytical performance of rat liver substrate slides in a real-life laboratory scenario by keeping in-house HEp-2 cell slides as reference standard so that in peripheral laboratories of resource limited developing countries this cost effective methods may be used for ANA screening.

Figure: Analytical evaluation of in-house rat liver substrate slides by keeping in-house HEp-2 cell slides as reference standard (n = 36)

METHODOLOGY

This cross sectional(concordance) study was carried out at Department of Immunology Armed Forces Institute of Pathology Rawalpindi, Pakistan for 6 months duration from January to June 2023. Study involved nonprobability sampling and sample size (n) was calculated using sensitivity specificity Arifin sample size calculator(online) by keeping 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 design effect final sample size of 36 was selected.[11]

Inclusion Criteria: Patients of either gender with age ranging from from 1 month to 85 years presenting with antinuclear antibodies positivity with common patterns including homogeneous, peripheral, coarse speckled, fine speckled, centromere and nucleolar pattern were included and ANA negative healthy controls were also included in the study.

Exclusion Criteria: Patient with hemolyzed, lipemic or icteric serum samples were excluded from study.

Study was formally approved from institutional ethical committee (IRB No-22/958) and written informed consent from patients and controls was taken.

Total 36 serum samples were selected, including ANA positive (n = 26) and ANA negative samples from healthy controls (n = 10). Samples were applied in 1:40 dilution on in-house HEp-2 cell slides which were used as reference standard12 and 1:10 dilution on in-house rat liver substrate slides and then subsequently stained with FITC labelled anti human IgG antibodies. Two experienced observers interpreted both in-house HEp-2 cell slides and in-house rat liver substrate slides independently without knowledge of interpretation by other observer. If two observers disagreed, a consensus was reached by mutual discussion.

For quality control, two controls (positive and negative) were tested in parallel on in-house HEp-2 cell slides and in-house rat liver substrate slides. Fluorescence was observed using immunofluorescent microscope BA-310 and observations were noted in predesigned proforma. Qualitative (gender, positivity/pattern, intensity) was expressed as frequency and percentages and quantitative variables (age) was expressed as Mean±SD. Data was analyzed using Microsoft Excel version 13.0 using visual reading of both independent observers on in-house HEp-2 cell slides as a reference standard and positive/negative results, staining patterns and intensities were compared between in-house HEp-2 cell slides and in-house rat liver substrate slides and overall percentage agreement and Cohen's k was used to determine degree of concordance. Interpretation of Cohen's k values were as follow: poor ≤ 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. p values < 0.05 were considered statistically significant and calculated using Fisher exact test.

RESULTS

Out of total 36 samples, in-house HEp-2 cell slides yielded 26 (72.22%) positive and 10 (27.78%) negative and in-house rat liver substrate slides yielded

27(75.00%) positive and 9 (25.00%) as negative. Using visual reading on in-house HEp-2 cell slides as the reference standard, relative sensitivity, specificity, and concordance of in-house rat liver substrate slides came out to be 100.00%, 90.00% and 97.22% (k = 0.92) respectively($p < 0.001$). For ANA positivity by both in-house HEp-2 cell slides and in-house rat liver substrate slides overall percentage agreement came out to be 96.42%. Overall percentage agreement for ANA negativity by both in-house HEp-2 cell slides and in-house rat liver substrate slides was 91.00%.For ANA intensity by both in-house HEp-2 cell slides and in-house rat liver substrate slides, the overall percentage agreement was 96.29%.For ANA patterns recognition by both in-house HEp-2 cell slides and in-house rat liver substrate slides, the overall percentage agreement came out to be 60.00%.Inter-observer agreement between two independent observers for ANA positivity, intensity and pattern determination on in-house HEp-2 cell slides and in-house rat liver substrate slides was 100%. The analytical performance of in-house rat liver substrate for differentiating positive/negative ANA results is summarized in Table-I.

Table-I: Analytical Performance of In-House Rat Liver Substrate Slides By Keeping In-House Hep-2 Cell Slides as Reference Standard (n = 36)

	In-House HEp-2 Cell Slides	
	Positive	**Negative**
In-House Rat Liver Substrate Slides		
Positive	26(72.22%)	1(2.78%)
Negative	0(0.00%)	9(25.00%)

Sensitivity = True Positive/(True Positive +False Negative)x100= 100%
Specificity = True Negative / (True Negative +False Positive)x100 = 90%
Positive Predictive Value = True Positive/ (True Positive+ False Positive)x100 = 96.29%
Negative Predictive Value = True Negative/ (True Negative +False Negative)x100 = 100%
Diagnostic Accuracy/ Overall percentage agreement = (True Positive +True Negative)/All Subjectsx100 = 97.22%
Overall agreement (oa) = (True Positive +True Negative)/All Subjects = 0.97
Probability of chance agreement(ca) = [(TP + FP)(TP + FN) + (FN + TN)(FP + TN)]/(TP + FP + FN + TN)2 = 0.611
Overall Concordance (Cohen's k) = (oa - ca)/(1 - ca) = 0.92
Fisher Exact Test Value (p) < 0.001

DISCUSSION

In present study by keeping visual reading on in-house HEp-2 cell slides as the reference, relative sensitivity, specificity, and concordance of in-house rat liver substrate slides came out to be 100.00%, 90.00% and 97.22% (k = 0.92) respectively($p < 0.001$). Finding by Hansson H *et al.* were consistent with present study who revealed that HEp-2 cells are better than rat liver substrate for ANA detection, because of low reactivity with normal sera and the ease of detection of fluorescence pattern. These 2 substrates for ANA-IIF detected identical population with suspected autoimmune diseases once the level of significant positive titer was adjusted to > 95% specificity for each substrate.[14]

Table-II: Concordance between in-house rat liver substrate and in-house HEp-2 cell slides (n = 36)

Immunofluorescence	Parameter(s)		
	Agreement (%)	**Cohen's k**	**Agreement**
Positivity	96.42%	0.65	Good
Negativity	91.00%	0.62	Good
Intensity	96.29%	0.65	Good
Pattern	60.00%	0.14	Poor

Findings by May YC *et al*. were also coherent with present study, they tested serum of 961 patients including 418 SLE patients concurrently on HEp-2 cells and rat liver. There was overall fair to moderate concordance between HEp-2 and rat liver ANA (kappa (k) = 0.35–0.79), titers (k = 0.34–0.79) and patterns (k = 0.35–0.93). Sensitivity of ANA for most SARD was highest when repeat testing using HEp-2cells, followed by HEp-2 and rat liver combined and when only HEp-2 or rat liver were used.[15]

Overall agreement between HEp-2 cells and rat liver substrate in Germany based study conducted by Kern P et al. was 75.44% within ±1 titer for both tissues. Agreement for positive and negative sera between the two tissues was 82.00%.[16]

In study conducted by Miller MH et al. ANA was performed on sera obtained from 141 patients with rheumatic disorders and 24 from healthy controls using HEp-2 cells and rodent liver substrates. Titers were higher using HEp-2 cells in almost all cases, majority by 1:5 dilutions. ANA positivity/negativity was mainly substrate independent, with few exceptions. Two patients with SLE showed positivity on HEp-2 only and rat liver only respectively; patterns were homogeneous. Thirteen out of 15 CREST patients had anti-centromere antibodies only detected on HEp-2 cells.[17]

Study conducted by Makni S *et al*. demonstrated the advantage of using HEp-2 cells in detecting

autoantibodies, especially when they are not detected on rodent liver sections, like anti-centromere antibodies. This substrate offers advantage of detecting antibodies not only directed against nuclear but also cytoplasmic antigens.[18]

Kumar Y et al. revealed few patients with SLE were negative on animal substrates (mouse kidney or rat liver) but positive on human substrate (HEp-2 cell). Due to variable sensitivity, reporting the type of substrate used is mandatory.[19]

There were some limitations to present study. First, because of pilot project present study included relatively less sample size and few specific ANA patterns, thus limiting our ability to precisely assess the accuracy of the rat liver substrate slides for these patterns only. Second, extractable nuclear antigen or the patient's disease status was disregarded when observing patterns by visual reading, as the aim of present study was to assess agreement between both HEp-2 cells and rat liver substrate slides under real-life working scenario. Finally, the probability of inter-observer bias cannot be ruled out in a single-center study, further multicenter studies will be required to overcome the reader's subjectivity, however in present study this bias is reduced by two observers reading results independently with inter-observer agreements of 100%. Evaluation in the context of above factors may be investigated in a future studies.

CONCLUSION

In conclusion, in-house rat liver substrate slides showed good agreement with in-house HEp-2 cell slides in distinguishing positivity/negativity and intensity. However, they still have limitation in pattern recognition. They can be used as valuable and cost effective substrate for ANA screening in resource limited conditions especially at peripheral setups.

Conflict of Interest: None.

Funding Source: None.

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 & MA: 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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