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Original Article

Pathology Section

Immunofluorescence on Formalin Fixed Paraffin Embedded Renal Tissue Sections: A Retrospective Study

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ABSTRACT

Introduction: Direct Immunofluorescence (DIF)/Routine Immunofluorescence (R-IF) on frozen sections is vital in the work-up of renal diseases. Sometimes, the unfixed sample may not be available for DIF or the sample may be inadequate. Paraffin Immunofluorescence (P-IF) can be used as a salvage technique in these situations. R-IF is more sensitive than P-IF in detecting Immunoglobulins (Ig) and complements. P-IF detects characteristic immunoglobulins and complements in the majority of glomerular diseases.

Aim: To evaluate the sensitivity of the P-IF in comparison to the gold standard R-IF in renal biopsies with proliferative and non proliferative glomerular diseases.

Materials and Methods: The present study was a retrospective study done on 52 selected cases, at St. John's Medical College, Bangalore, Karnataka, India, data collected from January to December 2016. Based on the clinical differential diagnoses, light microscopy and R-IF findings; selected panels of immunostains (IgG, IgA, IgM, C3, C1q) were done. Proteinase K was used for enzymatic digestion. Immunofluorescence intensity was scored by two pathologists independently. Any specific pattern of staining of atleast 1+ intensity was considered as positive on P-IF. Sensitivity, specificity and confidence intervals were estimated for P-IF.

Results: P-IF was done on a total of 52 selected cases. In this study P-IF showed 100% sensitivity for diagnosis of lupus nephritis, infection related glomerulonephritis, Henoch-Schönlein Purpura (HSP) nephritis and 78% for IgA nephropathy. Overall sensitivity in the diagnoses of common glomerular diseases studied was 90% (95% CI=78.97-96.80, p-value=0.025). It was less sensitive for detecting C3.

Conclusion: This retrospective study demonstrated that, P-IF has a good sensitivity for diagnosing common glomerulopathies like IgA nephropathy and lupus nephritis. P-IF is a good adjunct to R-IF testing with 100% specificity.

Keywords: Biopsy, Complement, Glomerular diseases, Immunoglobulins

INTRODUCTION

Direct immunofluorescence (DIF)/ routine immunofluorescence (R-IF) on frozen sections is vital in the aetiological work-up of renal diseases [1,2]. Routinely one core of renal biopsy is sent separately for R-IF in Michel's transport media/ normal saline. Sometimes the unfixed sample may not be available for DIF or the sample may be inadequate due to sampling of medullary tissue. P-IF performed on formalin fixed paraffin embedded tissue submitted for light microscopy can be used as a salvage technique in these situations. Formalin fixation causes cross linking of globular proteins and preserves the secondary structure of proteins in tissues [3]. It is difficult to detect immunoglobulins and complements in formalin fixed paraffin embedded tissue sections. Antigen retrieval helps antibodies to bind to unmasked antigens for detection [4]. P-IF with antigen retrieval by enzyme treatment was described long ago [5-9]. However, only in the recent past, it is put into use as an adjunct to R-IF on fresh frozen tissue [10]. In P-IF procedure enzymes like trypsin, pronase, pepsin and Proteinase K are used in enzymatic digestion and followed by direct or indirect method of IF. Heat treatment can also be used for the P-IF [11]. R-IF is more sensitive than P-IF in detecting immunoglobulins and complements. P-IF detects characteristic immunoglobulins and complements in the majority of glomerular diseases, such as, IgA nephropathy, lupus nephritis, and infection related glomerulonephritis and membranous nephropathy [7-9]. The concordance rate between R-IF and P-IF reported in the literature varies from 83-100% [8-10,12-14]. The present study was undertaken to evaluate the sensitivity and specificity of the P-IF technique in the diagnosis of common proliferative as well as non proliferative glomerular diseases.

MATERIALS AND METHODS

The present study was a retrospective study done in the Department of Pathology, St. John's Medical College, Bangalore, Karnataka, India, data collected from January to December 2016. P-IF was performed on selected cases from 2018-2021. Analysis of the data done from January to April 2022. This study was approved by the Institutional Ethics Committee (IEC), St. John's Medical College and Hospital, Bangalore (IEC Study Ref. No.174/ 2017).

Inclusion criteria: Renal biopsies from cases of all age groups with adequate cortical tissue were included in the study.

Exclusion criteria:

1. Renal biopsies with inadequate cortical tissue/ diffuse glomerulosclerosis.
2. Paraffin block not available.

Sample size calculation: To estimate sensitivity of 80% with 15% relative precision and 95% Confidence Interval (CI), sample size required was 42, but final sample was taken as 52.

P-IF procedure was validated, by doing it on two cases of lupus nephritis with full house positivity.

P-IF Procedure

1. 3 to 4 µ sections were cut from the formalin fixed paraffin embedded tissue block. Sections were taken on poly -L-Lysine (PLL) coated slides. Each slide was labelled appropriately.
2. Deparaffinisation was done in a slide incubator for 1 hour and later kept in xylene for 10 minutes.
3. Slides were immersed for 30 minutes in Tris EDTA buffer (pH=9) at room temperature.

4. Proteinase K (ready to use from Dako) was used for enzymatic digestion. Slides were kept in a moisture chamber on a level surface. PAP (Peroxidase Anti-Peroxidase/buffer) pen was used to circle the tissue. One or two drops of Proteinase K solution pipetted to completely cover the sections. Incubated for 45 minutes.
5. Enzymatic digestion was stopped by transferring the slides to Tris EDTA buffer at 4°C and left for 40 minutes.
6. Slides were rinsed in PBS buffer for 10 minutes.
7. Fluorescein isothiocyanate (FITC) conjugated polyclonal rabbit antibodies (Dako) were applied and incubated for two hours in a moist chamber in the dark. (All antibodies were used in dilution of 1:30).
8. Rinsed in PBS buffer and mounted in glycerol.
9. Slides were examined in fluorescence microscope and representative images were captured.

Based on the clinical differential diagnoses, light microscopy and R-IF findings; selected panel of immunostains (IgG, IgA, IgM, C3 and C1q) were performed on P-IF.

Following parameters were assessed on P-IF: The immunofluorescence intensity of P-IF was evaluated and scored on a semi-quantitative scale of 0-3+ (0-absent, 1+-mild, 2+- moderate, 3+- strong), by two pathologists independently and were blinded to R-IF findings. Intensity and location of the immune deposits on P-IF were compared with the corresponding R-IF. Any specific pattern of staining of atleast 1+ intensity was considered as positive on P-IF. Trace positivity was taken as negative. P-IF results were categorised as positive (1+ and above) or negative (score 0) for analysis. R-IF is the gold standard test for detecting immunoglobulins and complements. The Concordance rate between P-IF and R-IF was estimated.

STATISTICAL ANALYSIS

Sensitivity, specificity, 95% confidence intervals were estimated for P-IF. McNemar's test was used to calculate the p-value. Categorical variables were expressed as numbers and percentages. Sensitivity, specificity and confidence intervals were estimated for each immunostain (IgG, IgA, IgM, C3 and C1q) on P-IF irrespective of the glomerular disease.

RESULTS

The P-IF was performed on 52 selected cases. Concordance rate of P-IF with R-IF in various glomerular diseases was calculated [Table/Fig-1]. Among the common glomerulopathies studied by P-IF, 100% sensitivity was observed for lupus nephritis, infection related glomerulonephritis and HSP nephritis. No false positive staining was observed in cases of diabetic nephropathy, minimal change disease and focal segmental glomerulosclerosis. For IgA nephropathy and membranoproliferative glomerulonephritis, sensitivity of 78% and 75%, respectively was noted.

Glomerular disease category	Number of cases	% P-IF cases concordant with R-IF
IgA Nephropathy	9	7/9 (78%)
Lupus nephritis	9	9/9 (100%)
Infection related glomerulonephritis	6	6/6 (100%)
Membranous nephropathy	6	4/6 (67%)
Diabetic nephropathy	6	6/6 (100%)
Minimal change disease	6	6/6 (100%)
Focal segmental glomerulosclerosis	3	3/3 (100%)
Membranoproliferative glomerulonephritis	4	3/4 (75%)
Henoch-Schönlein purpura nephritis	2	2/2 (100%)
Anti GBM disease	1	1/1 (100%)
Over all	52	47/52 (90%, 78.97-96.80) p-value=0.025

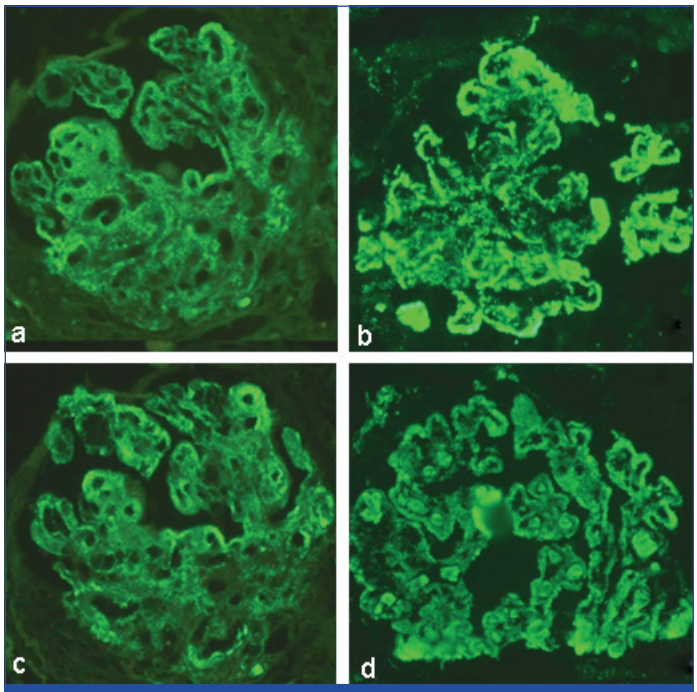
[Table/Fig-1]: Concordance rate of P-IF with R-IF in various glomerular diseases.
R-IF: Routine immunofluorescence; P-IF: Paraffin immunofluorescence
McNemar's statistical test was applied

Sensitivity and specificity of each immunostains on P-IF in comparison to gold standard R-IF is depicted in [Table/Fig-2]. Of the immunostains performed none showed non specific/ false positive staining. IgG, IgA, IgM and C1q showed better sensitivity than C3.

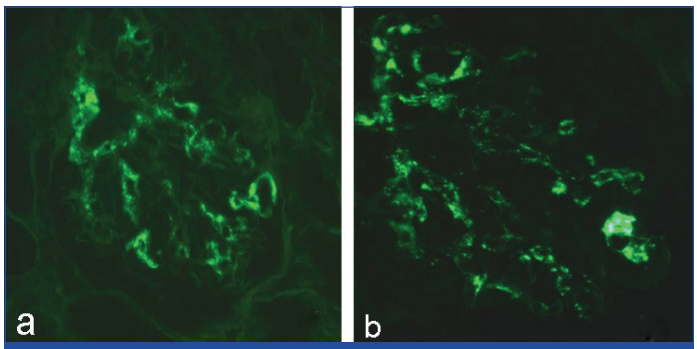
Immunostain	Sensitivity (confidence interval)	Specificity (confidence interval)	p-value
IgG	78.26% (56.30% to 92.54%)	100.00% (29.24% to 100.00%)	0.025
IgA	83.33% (58.58% to 96.42%)	100.00% (15.81% to 100.00%)	0.083
IgM	85.71% (57.19% to 98.22%)	100.00% (47.82% to 100.00%)	0.157
C3	54.55% (32.21% to 75.61%)	100.00% (29.24% to 100.00%)	0.001
C1q	84.62% (54.55% to 98.08%)	100.00% (47.82% to 100.00%)	0.157

[Table/Fig-2]: Sensitivity and specificity of each immunostain on P-IF.
R-IF: routine immunofluorescence, P-IF: Paraffin immunofluorescence;
*Online OMNI calculator was used to calculate the p-value using standard McNemar's test

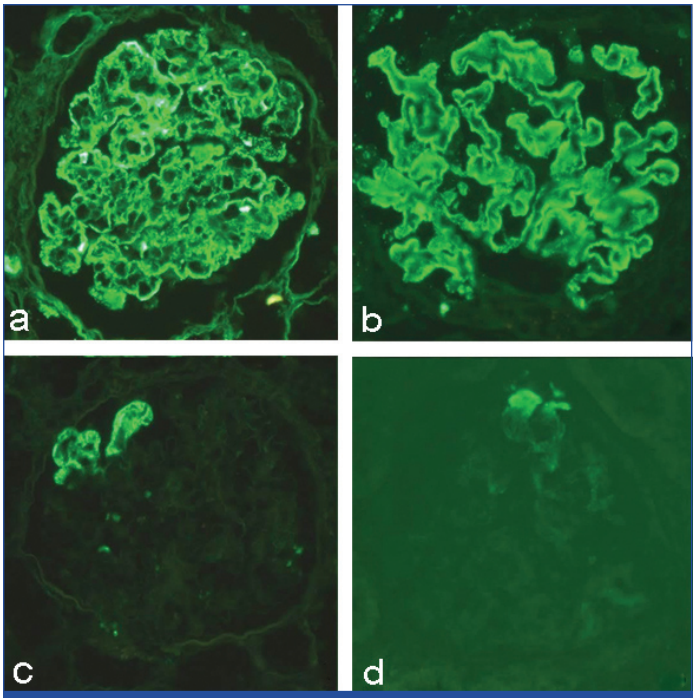
[Table/Fig-3-5] shows P-IF and R-IF of Lupus nephritis, IgA nephropathy, membranous nephropathy and focal segmental glomerulosclerosis.



[Table/Fig-3]: Left panel P-IF images and right panel corresponding R-IF images: IgG (a,b) and C1q (c,d) positivity in mesangium and capillary loops in a case of lupus nephritis.



[Table/Fig-4]: Left panel P-IF image and right panel corresponding R-IF image: Case of IgA nephropathy with mesangial, granular IgA positivity (a,b).



[Table/Fig-5]: Left panel P-IF images and right panel corresponding R-IF images: IgG positivity in capillary loops in a case of membranous nephropathy (a,b). IgM segmental deposits in FSGS (c,d).

of P-IF in membranous nephropathy and anti-GBM disease. They also observed reduced sensitivity of P-IF for C3 immunostain [10]. In this study, sensitivity of 67% for membranous nephropathy [Table/Fig-5] and 55% for C3 immunostain, was observed.

In cases of diabetic nephropathy, minimal change disease and focal segmental glomerulosclerosis no non specific staining was observed. They were clearly negative. Linear IgG staining and segmental sclerosis [Table/Fig-5] were well demonstrated on P-IF in diabetic nephropathy and focal segmental glomerulosclerosis cases, respectively. Singh G et al., observed that P-IF interpretation was possible in 87% (214/ 246) [13].

On P-IF granular staining, pattern of immunoglobulins and complements were less evident and appeared smudgy [16]. Similar staining pattern was observed in the present study.

Apart from its use as a salvage technique, Messias NC et al., have shown the unmasking effect of P-IF on immune complex deposits in C3 dominant glomerulonephritis, which precludes unnecessary labeling of these cases as C3 glomerulopathies [17]. Nasr SH et al., demonstrated the usefulness of P-IF in dysproteinaemia associated with renal diseases. P-IF is more sensitive than R-IF in light chain Fanconi syndrome [10]. Membranous-like Glomerulopathy with Masked IgG kappa Deposits (MGMID) and Membranoproliferative Glomerulonephritis (MPGN) with masked monotypic Ig deposits require P-IF for diagnosis [18-20]. Recent studies have applied

Studies comparing P-IF (enzyme treatment method) and R-IF	Sample size	Enzyme used	Results
Qualman SJ and Keren DF [6]	52	Trypsin	Immunoglobulins and fibrinogen detected in 90% and complements in 75%
Choi YJ and Reiner L [8]	21	Trypsin	21/21 (100%) Concordant for the immunoglobulins characteristic of a particular glomerular disease. No reaction for complement
Fogazzi GB et al., [9]	28	Pronase	28/28 (100%) Concordant for the immunoglobulins characteristic of a particular glomerular disease. Less reaction for complement
Nasr SH et al., [10]	71	Pronase	59/71 (83%) concordant
Wagrowska-Danilewicz M and Danilewicz M [12]	66	Proteinase K	IgA-57%, IgM-44%, IgG-74%, C3-52%. Included IgA nephropathy, membranous nephropathy and lupus nephritis
Singh G et al., [13]	37	Proteinase K	35/37 (95%) concordant, only 2 cases with >2+ difference in intensity of staining
Solanki R et al., [14]	50	Proteinase K	92% concordant
Alwahibi NY et al., [15]	101	Proteinase K	Sensitivity: IgA- 45.6%, IgG -69.4% and IgM -52.8% Specificity: IgA -87.9%, IgG -87.2% and IgM- 77.1%
Current study	52	Proteinase K	47/52 (90%) concordant

[Table/Fig-6]: Studies on renal biopsies comparing P-IF and R-IF [6,8-10,12-15].
R-IF: routine immunofluorescence, P-IF: Paraffin immunofluorescence

DISCUSSION

The DIF testing on frozen tissues is essential in the aetiological work-up of renal biopsies [1,2]. R-IF is essential in the diagnosis of glomerular diseases and it requires an unfixed sample in Michel's transport media/ normal saline. In certain situations, separate unfixed sample may not be available or the sample may be inadequate due to lack of glomeruli. In such instances, P-IF can be performed on paraffin sections as a salvage technique. Many studies have been performed P-IF on renal tissue sections using different enzymes like trypsin, pronase and proteinase K for enzymatic digestion. In the present study, proteinase K was used and the P-IF method described by Singh G et al., was followed [13]. P-IF staining intensity and pattern matched with R-IF, in most of the cases. The concordance was good when the immune complex deposits were bright and abundant (3+) on R-IF. P-IF, a salvage technique has fair sensitivity in the diagnoses of common glomerulopathies. Studies have shown that the R-IF and P-IF results were in agreement for immunoglobulins, characteristic of a particular glomerular disease [Table/Fig-6] [6,8-10,12-15]. In the present study, 100% sensitivity was observed for diagnosis of lupus nephritis [Table/Fig-3], infection related glomerulonephritis, Henoch-Schönlein purpura nephritis and 78% for IgA nephropathy [Table/Fig-4]. Nasr SH et al., have shown slightly lower sensitivity P-IF for light chain detection in amyloidosis and dysproteinaemia in extrarenal locations, as it is more sensitive than immunohistochemistry [21-23].

As a salvage technique, P-IF exhibits fairly good sensitivity in diagnosing immune-complex mediated glomerular diseases, especially when the immune deposits are abundant. Future studies on larger samples, encompassing various glomerular diseases with weak to abundant immune deposits, are necessary.

Limitation(s)

Since, renal biopsies with adequate cortical tissue on paraffin sections were retrospectively selected for the study; there could have been a selection bias, as glomerular diseases with better defined and evolved morphological patterns might have been favored. Chances of false negative results are higher in early glomerular diseases. Secondly, limited number of common glomerular diseases were included in the study. Also, kappa and lambda light chain immunostains were not performed.

CONCLUSION(S)

In the present study, sensitivity of 90% (95% CI=78.97-96.80, p-value=0.025) was observed for P-IF in diagnosis of various common glomerular diseases and false positive results were

none. To conclude, P-IF is a good salvage technique, with 100% specificity.

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