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Utility of Direct Immunofluorescence Using Proteinase-k for Antigen Retrieval in Formalin-Fixed, Paraffin-Embedded Kidney Biopsy Sections

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ABSTRACT

Background: Immunofluorescence on frozen sections (IF-F) is crucial for diagnosing immune-complex-related glomerular diseases. Immunofluorescence on paraffin block (IF-P) is applicable when IF-F is unfeasible. The efficacy of IF in formalin-fixed, paraffin-embedded (FFPE) kidney biopsies with proteinase-K was evaluated.
Methods: This prospective study encompassed a total of 100 cases (control-set = 30, study-set = 70). Proteinase-K was employed for enzymatic digestion to facilitate antigen retrieval on FFPE blocks. The sensitivity and specificity of detecting Immunoglobulin G (IgG), Immunoglobulin A (IgA), Immunoglobulin M (IgM), C3, C1q, kappa, and lambda with IF-P were compared to IF-F.
Results: In the control group, the total positive for all antibodies, excluding C3c, on IF-P was comparable to that on IF-F, demonstrating a sensitivity of 100%. The sensitivity for C3c was 54.26%. Specificity exceeded 90% for all antibodies, with the exception of C1q and lambda. The intensities on IF-F were brighter than those on IF-P. Of the 70 study-set cases, the IF-P results were consistent with the light microscopy (LM) diagnosis in 66 cases. However, IF-P was negative in one case of classic membranous nephropathy and 3 cases of infection-related glomerulonephritis diagnosed on LM.
Conclusion: Immunofluorescence on paraffin block is optimally utilized as a rescue strategy or to enhance the sensitivity of immunoglobulin detection in instances where IF-F is ineffective.
Keywords: Proteinase-k, direct immunofluorescence, paraffin immunofluorescence, antigen retrieval, kidney biopsy

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INTRODUCTION

The interpretation of kidney biopsy involves a combined analysis of light microscopy (LM), immunofluorescence (IF), and electron microscopy (EM) in the light of clinical syndromes. Though the indications for EM in kidney biopsy are limited, immunofluorescence is essential for accurate diagnosis in every biopsy. This necessitates an additional fresh tissue core stained with immunoglobulins, complements, and light chains for the diagnosis of various immune-related and non-immune glomerular and tubulointerstitial diseases. Immunofluorescence continues to serve as a crucial research instrument for investigating a range of antigens to elucidate disease pathogenesis. The adequacy of the core for IF examination requires a specimen of cortex, roughly 20% of the biopsy, comprising more than 5 glomeruli.[1] Owing to technical issues or patient-related considerations, a tissue core for immunofluorescence may occasionally be unavailable or deemed insufficient. Under these circumstances, IF on formalin-fixed, paraffin-embedded (FFPE) tissue may be conducted to preclude the necessity of a repeat biopsy. Antigen retrieval from paraffin-embedded tissue is the most critical step, utilizing proteolytic enzymes such as trypsin, pronase, and proteinase K.[2-6] This study aims to analyze the utility of direct IF in FFPE kidney biopsies.

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MATERIAL AND METHODS

This was a prospective observational study conducted on FFPE blocks of kidney biopsies of selected glomerulopathies between January 2022 and June 2024. Informed consent was acquired before the kidney biopsy and the study has received approval from the Nizam's Institute of Medical Sciences ethics committee, as indicated by review letter number EC/NIMS/3158/2023, dated June 17, 2023.

The study period had a total of 100 cases (control-set = 30, study-set = 70). The controls comprised known cases of membranous nephropathy (MN), membranoproliferative glomerulonephritis (MPGN), IgA nephropathy, infection-related glomerulonephritis (IRGN), diabetic nephropathy (DN), lupus nephritis (LN), minimal change disease (MCD), chronic active antibody-mediated rejection, and crescentic glomerulonephritis (immune-complex mediated). The control-set includes cases evaluated using both IF-F and IF-P on the tissues (formalin-fixed biopsy core and another core sent in normal saline) obtained from the same patient. The inclusion criteria for study-set cases were inadequate biopsy tissue for IF-F, exposure of IF-sample to formalin, negative result in IF-F, and the necessity for revision of the IF report following the acquisition of electron microscopic report. The exclusion criterion was insufficient tissue in the FFPE block.

Protocol for Proteinase-IF

Proteinase-K was employed for enzymatic digestion to facilitate antigen recovery on FFPE blocks. Four-micron sections of the biopsy were placed on poly-L-lysine coated slides and incubated at 37°C overnight. Deparaffinization was performed through 3 successive immersions in xylene for 10 minutes each, followed by 1 immersion in 100% ethyl alcohol for 5 minutes, and subsequent changes in 70% and 50% ethyl alcohol for 5 minutes each. Slides were rehydrated by immersion in distilled water (20 dips) and thereafter maintained in Tris buffer (pH 9.0) at 37°C for 20 minutes. Incubation with proteinase-K at a concentration of 0.25 mg/mL was conducted at 37°C for 7 minutes. Following washing with Tris buffer (pH 9.0), the slides were incubated at 4°C for 20 minutes. Fluorescein isothiocyanate (FITC) conjugated polyclonal antibodies targeting immunoglobulins (IgG, IgA, IgM), complements (C3, C1q), and light chains (kappa, lambda) were utilized according to the manufacturer's guidelines. Incubation occurred for 2 hours in a moist, humidified chamber. Slides were washed in phosphate buffer solution and mounted with aqueous phosphate buffer glycerin. Slides were analyzed using a dark field fluorescent microscope (Zeiss). DIF intensity was assessed semi-quantitatively using a scale of 0 (negative), 1+ (mild), 2+ (moderate), and 3+ (strong positivity), and compared with DIF interpretation on fresh tissue in the control set.

Statistical Analysis

Ranges, ratios, percentages, and medians were calculated for descriptive analysis. Sensitivity and specificity (with 95% confidence intervals) were computed using 2 × 2 contingency tables for IgG, IgA, IgM, C3, C1q, kappa, and lambda using the IF-P technique among the control set only, as the study set lacks IF-F results.

RESULTS

A total of 30 control cases were subjected to routine IF and IF-P, alongside 70 study-set cases. The demographic characteristics and clinical manifestations of these patients are detailed in Table 1.

The primary indication for IF-P in the cases examined in this study was inadequate biopsy tissue for IF-F (n = 45), followed by exposure of the IF sample to formalin (n = 12), negative IF-F

MAIN POINTS

- Proteinase-K was used for enzymatic digestion to retrieve antigens from formalin-fixed, paraffin-embedded (FFPE) blocks when frozen-immunofluorescence (IF) failed due to inadequate biopsy tissue, exposure of the IF sample to formalin, or for the revision of the IF report following electron microscopic findings.
- A total of 100 cases were included (control-set = 30, study-set = 70). The findings demonstrated complete (100%) sensitivity of this method for IgG, IgA, IgM, C1q, kappa, and lambda. The sensitivity for C3c was 54.26%.
- Frozen-IF exhibited greater sensitivity than IF-paraffin in the detection of c3c. The intensities on frozen-IF were more luminous than those on IF-paraffin.
- In addition to the diminished detection of C3c, the background paraffin wax on the slide may complicate the interpretation of the results.
- It is concluded that proteinase-K enzyme-treated FFPE tissue is suitable for demonstrating immunoglobulins and complement deposits when frozen IF is impractical.

Table 1. Demographic Parameters and Clinical Presentations

	Study-set (n = 70)	Control-set (n = 30)
Age (Range)	11-78 Years	20-61 Years
Male: Female	28:32	11:19
Clinical presentations		
Nephrotic syndrome	19	11
Nephritic syndrome	23	12
RPKF	18	5
CKD	6	1
AKI	4	1
Lab parameter (median)		
Serum creatinine mg/dl	0.22 to 55.01 (2.55)	0.18 to 5.12 (1.78)
UPCR	0.13 to 18.37	1.06 to 4.12 (3.51)
Blood urea nitrogen nitrogen, mg/dl	18 to 105 (63.5)	22 to 95 (54.50

AKI, acute kidney injury; CKD, chronic kidney disease; RPKF, rapidly progressive kidney failure; UPCR, urine protein to creatinine ratio.

results (n = 12), and revision of the IF report subsequent to the electron microscopic report (n = 1).

The immunoreaction to all the antibodies of the control cases, on both IF-F and IF-P, together with their intensities, is presented in Table 2. The overall positivity for all antibodies, excluding C3c, on IF-P was similar to that on IF-F, exhibiting a sensitivity of 100% (Figure 1). The sensitivity for C3c was merely 54.26%, indicating that C3c yielded false-negative results in few cases, while IF-F demonstrated greater sensitivity than IF-P in detecting C3c across all the disease categories (Table 3). Also, the intensities on IF-F were brighter as compared to those on IF-P (Figure 2). Specificity exceeded 90% for all antibodies, with the exception of C1q and lambda.

Of the 70 study-set cases, the IF-P results were consistent with the LM diagnosis in 66 cases (as shown in Table 4). Nonetheless, among the study-set cases, IF-P was negative in 1 case of classic MN and three cases of IRGN diagnosed on LM.

DISCUSSION

Immunofluorescence is a crucial diagnostic research method for assessing kidney biopsies. Direct IF-F is regarded as the gold standard. Immunofluorescence on fresh frozen sections aids in the definitive diagnosis of anti-glomerular basement membrane (GBM) disease, immune-related GN, light-chain diseases, and pauci-immune glomerulonephritis. It helps in early stages of MN, wherein the glomerular basement membrane thickness is not discernible on LM, as well as in focal segmental glomerulosclerosis characterized by IgM deposits.[7] Nonetheless, IF-F has limitations, including the necessity for a specialized laboratory equipped with a cryostat and fluorescence microscope, as well as a skilled team of technicians and a specialist pathologist. Additionally, there are challenges related to transportation (which must occur on ice and reach the laboratory within one hour of the biopsy procedure) and the preservation of the delicate, unfixed kidney biopsy, which is susceptible to desiccation and becomes fixed upon exposure to formalin vapors. This necessitates the exploration of alternative techniques that can provide assistance when IF-F fails. This brings us to the objective of this study, which is to assess the utility of DIF in FFPE kidney biopsies.

This study demonstrated a 96.67% (29/30) concordance between IF-F and IF-P results, comparable to prior studies utilizing proteinase-K.[8,9] Tables 5 and 6 show discrepancies among the findings of several studies which are likely attributable to the specific enzymes employed for the proteolytic digestion of paraffin-embedded tissues. Mubarak M et al[11] discovered that IF-P staining exhibited lower sensitivity and intensity compared to IF-F staining across three specific antigen retrieval methods: heat-induced antigen retrieval with Tris-buffer (HIAT-TBS), HIAT with citrate-buffer, and pronase. Among the 3 approaches, TBS-based HIAT was deemed the most beneficial. The IF staining yield, in terms of overall positive and enhanced intensity, was superior to the other two procedures.

Notwithstanding the limited sensitivity, Choi YJ et al[2] determined that IF-P can be utilized to diagnose immune complex-mediated glomerular diseases in the majority of situations. Consistent with the findings of Mohammadzadeh S et al[9] and Mubarak M et al[11], it was observed that the intensities on IF-F were more luminous than those on IF-P.

Van der Ven et al[12] demonstrated that IF-P with bacterial proteinase XXIV digestion exhibits greater sensitivity compared to pronase digestion, with less background observed. Proteinase XXIV exhibited greater sensitivity in kidney

Table 2. Over-all Positivity and Intensity of Various Immunoreactants in Controls According to Different Methods

Immunoreactants	Method	Negative	+1	+2	+3	Total positive (%)
IgG (n = 30)	IF-F	07/30	03/30	05/30	15/30	23 (76.67)
	IF-P	07/30	05/30	12/30	6/30	23 (93.33)
IgM (n = 30)	IF-F	29/30	01/30	–	–	01 (03.33)
	IF-P	29/30	01/30	–	–	01 (03.33)
IgA (n = 30)	IF-F	20/30	02/30	2/30	06/30	10 (33.33)
	IF-P	19/30	03/30	07/30	1/30	11 (36.67)
C3C (n = 30)	IF-F	06/30	3/30	05/30	16/30	24 (80.0)
	IF-P	17/30	03/30	03/30	07/30	13 (43.34)
C1q (n = 30)	IF-F	18/30	02/30	03/30	07/30	12 (40.0)
	IF-P	12/30	02/30	11/30	05/30	18 (60.0)
Kappa (n-30)	IF-F	18/30	01/30	05/30	06/30	12 (40.0)
	IF-P	17/30	04/30	05/30	04/30	13 (43.34)
Lambda (n-30)	IF-F	16/30	01/30	06/30	07/30	14 (46.67)
	IF-P	12/30	03/30	09/30	06/30	18 (60.33)

IgA, Immunoglobulin A; IgG, Immunoglobulin G; IgM, Immunoglobulin M.

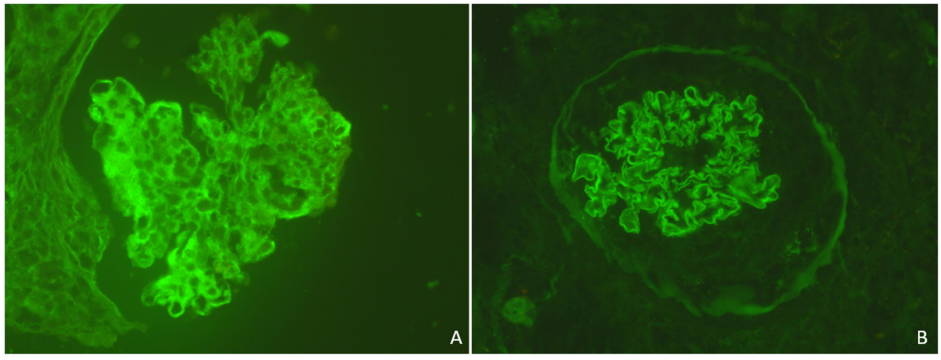

Figure 1. (A) IgG on IF-F showing 3+ granular basement membrane staining pattern. (B) Same case on IF-P showing a similar 3+ granular basement membrane staining pattern.

samples with lupus nephritis when staining for IgG and C1q, in contrast to pronase.

It was found that C3c yielded false-negative results in several instances and that IF-F exhibited greater sensitivity than IF-P in detecting C3c across all disease categories. This may result in diagnostic inaccuracies in the identification of C3GN and other immune-mediated glomerulonephritis, such as IRGN.

Fogazzi et al[3], Nasr et al[4], and Mohammedzadeh S et al[9] also effectively exhibited immunoglobulins in the majority of diseases. Nonetheless, staining with complements was not as effective in these cases. Nasr et al[4] noted that IF-P exhibited negative to mild staining for C3 in cases when C3 is the predominant deposit. They deemed IF-P adequate in illustrating light chain restriction in tubulointerstitial disorders, including myeloma cast nephropathy and light chain proximal tubulopathy.

Nada R et al[6] also found proteinase-K digestion efficient in both immunoglobulins and complements in cases when the quantity of immune deposits is modest to plentiful. Nonetheless, early cases with few deposits are challenging to diagnose using IF-P. Patchy staining was observed in specific instances. In certain instances, they observed a blurred, less granular staining pattern compared to that observed on IF-F, which could hinder the diagnosis of fibrillary glomerulonephritis, where a blurred/smudgy staining is a defining trait. The authors concluded that IF-P is an unreliable approach for identifying anti-GBM disease due to the indistinct staining, which prevented the identification of a linear staining pattern in 2 cases of anti-GBM. However, in autopsy cases of anti-GBM disease, linear staining was observable on thicker sections.

The analysis identified one case of MN (among the control-set cases) that was negative on standard IF-F but shown positivity on IF-P. This may be attributed to masked antigens, as noted by Messias et al.[13] The IF-P approach was deemed effective for assessing masked paraprotein and immune complex deposits. The light chain crystals in light chain proximal tubulopathy were observed solely upon enzyme digestion. The diagnosis of membranous-like glomerulonephritis with masked IgG-kappa deposits (MGMIDK), a novel entity initially described by Larsen et al[14], as well as MPGN with light chain restriction and MPGN with mixed essential cryoglobulinemia, was facilitated, preventing misdiagnosis as C3 glomerulonephritis. It is advised that cases of C3 glomerulonephritis identified using IF-F undergo further IF-P, to prevent misinterpretation and minimize superfluous

Table 3. Sensitivity and Specificity for IgG, IgA, IgM, C3, c1q, Kappa and Lambda by IF-P Method

IF-F/IF-P	A (+/+)	B (−/+)	C (+/−)	D (−/−)	Total cases	Sensitivity A/A + C (%)	Specificity D/B + D (%)
IgG	23	0	0	7	30	100.0	100.0
IgA	10	01	0	19	30	100.0	95.00 (±0.04)
IgM	1	0	0	29	30	100.0	100.0
C3c	13	0	11	06	30	54.16 (±0.09)	100.0
C1q	12	06	0	12	30	100.0	66.67 (±0.09)
Kappa	12	01	0	17	30	100.0	94.44 (±0.04)
Lambda	14	04	0	12	30	100.0	75.01 (±0.08)

IF-F, Immunofluorescence on frozen sections; IF-P, Immunofluorescence on paraffin block; IgA, Immunoglobulin A; IgG, Immunoglobulin G; IgM, Immunoglobulin M.
()-95% confidence interval. For a sensitivity or specificity value of 100%, the 95% confidence interval would range from 100% to 100%.

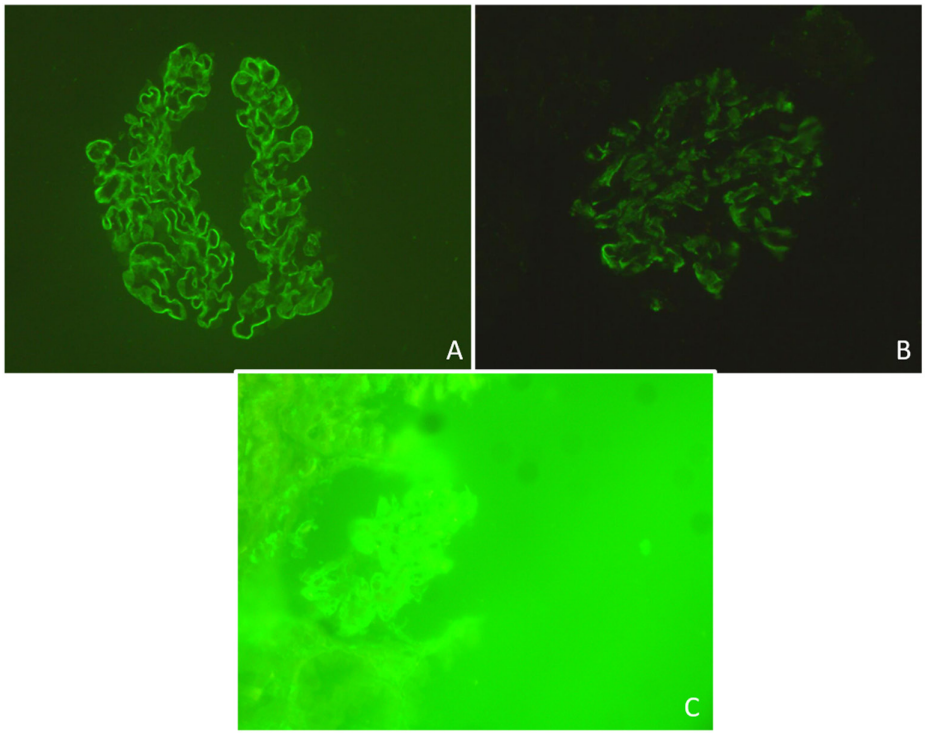

Figure 2. (A) IgG on IF-F showing 3+ granular basement membrane staining pattern. (B) Same case on IF-P showing a 1+ faint smudgy to granular basement membrane staining pattern. (C) The background staining seen in some cases during IF-P.

investigations into complement abnormalities. Furthermore, every instance in which the IF-F findings are inconsistent with the ultrastructural findings should be subjected to IF-P analysis.

Numerous investigations have shown false-positive staining on the surface and within capillary lumina, attributable to serum adsorption resulting from fixation.[13,15] This was similarly

Table 4. Comparison of Cases in This Study with Other Studies, in Respect to Various Kidney Pathologies

		Nada R et al[6] n = 75		Singh G et al[15] n = 119		Present Study n = 70	
	Light Microscopy Diagnosis [Cases]	Positive	Negative	Positive	Negative	Positive	Negative
1	Membranous nephropathy	4		9	2	8	1
2	IgA Nephropathy	3		39	–	12	–
3	Diabetic nephropathy	–		–	–	–	4
4	Infection related glomerulonephritis	–		11	1	8	3
5	Membranoproliferative glomerulonephritis	46	6	30	2	5	–
6	ANTI GBM			–	–	1	–
7	Minimal change disease	–		–	–	–	3
8	DDD	2		–	–	–	–
9	C3 glomerulonephritis	4		–	–	–	–
10	Lupus nephritis	2		24	1	18	–
11	Crescentic GN	–	–	–	–	1	1
12	Others (Hypertension, Chronic Interstitial nephritis)	–	–	–	–	–	5
	Total	61	6	113	6	53	17

Anti GBM, Anti Glomerular Basement Membrane Disease; DDD, Dense deposit disease; GN; glomerulonephritis.

Table 5. Comparison of Percentage Concordance Between IF-P and IF-F, with Other Studies

Glomerular Disease Category	Malipatel R et al[8] (%)	Solanki R et al[10] (%)	Current Study (%)
IgA Nephropathy	7/9 (78)		2/2 (100)
Lupus nephritis	9/9 (100)	8/9 (88.8)	9/9 (100)
Infection related glomerulonephritis	6/6 (100)		4/4 (100)
Membranous nephropathy	4/6 (67)	7/8 (87.5)	5/6 (83.34)
Diabetic nephropathy	6/6 (100)		2/2(100)
Minimal change disease	6/6 (100)	3/3 (100)	3/3 (100)
Focal segmental glomerulosclerosis	3/3 (100)	1/1 (100)	–
Membranoproliferative glomerulonephritis	3/4 (75)		1/1 (100)
Henoch-Schönlein purpura nephritis	2/2 (100)	4/4 (100)	–
Anti GBM disease	1/1 (100)		–
C3 glomerulonephritis	–	3/5 (60)	–
Immune-complex mediated glomerulonephritis	–	19/19 (100)	2/2 (100) - crescentic glomerulonephritis
IgM nephropathy	–	1/1 (100)	–
Chronic active Antibody mediated Rejection	–	–	1/1 (100)
Over all	47/52 (90)	46/50 (92)	29/30 (96.67)

GBM, Glomerular basement membrane; IgA, Immunoglobulin A; IgG, Immunoglobulin G.

noted in the present study and was more evident in inadequately digested tissue. Therefore, it is essential to examine the stained portion of the glomerulus to avoid conflating intracapillary staining with immune-complex deposition.[16] Moreover, Mohammedzadeh S et al[9] identified an increased background in certain cases of IgG utilizing the IF-P method. Various optimization methods were employed to mitigate background noise, although none proved effective. Certain studies ascribe this robust background to substantial thick sections or the existence of endogenous activity.[17]

Table 6. Studies Using Different Enzyme Treatments, Comparing IF-P and IF-F

	Sample Size	Enzyme Used	Results
Choi YJ and Reiner L[2]	21	Trypsin	21/21 (100%) Concordance for Igs. No reaction for complement.
Fogazzi GB et al.,[3]	28	Pronase	28/28 (100%) Concordant for Igs. Less reaction for complement.
Nasr SH et al.,[4]	71	Pronase	59/71 (83%) concordant
Alwahibi NY et al.,[5]	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%
Malipatel R et al[8]	52	Proteinase K	47/52 (90%) concordance Sensitivity: IgG - 78.26%, IgA - 83.33%, IgM - 85.71%, C3 - 54.55%, C1q - 84.62%. Specificity: 100.00% (IgG, IgA, IgM, C3, C1q)
Mohammadzadeh S et al[9]	51	Proteinase K	49/51 (96%) concordance Sensitivity: IgG - 93.1%, IgA - 76.9%, IgM - 63.6%, and C3 - 33.3%, Specificity of IgG - 100%, IgA - 97.3%, IgM - 95%, and C3 - 100%.
Present study	30	Proteinase K	29/30 (96.67%) concordance Sensitivity: 100% (IgG, IgA, IgM, C1q, kappa, lambda) and 54.16% - C3c. Specificity: 100.00% (IgG, IgM, C3c), 95% - IgA, 66.67% - C1q, 94.44% - kappa, 75% -lambda

IgA, Immunoglobulin A; IgG, Immunoglobulin G; IgM, Immunoglobulin M.

Table 4 presents a comparative analysis of the findings and those of other published studies concerning various kidney diseases. Out of the 70 study-set cases, IF-P was identified as false negative in 4 cases with typical LM morphology and corresponding clinical features. The erroneous negativity in IRGN was attributable to the inadequate sensitivity of IF-P for C3c. Inadequate fixation or processing coupled with defective antigen retrieval may account for negative IgG in MN. Improper paraffin block storage conditions and extended archival durations can adversely affect the output of paraffin immunofluorescence.

Notwithstanding the aforementioned disadvantages, the benefits of IF-P include a unified core for both LM and IF, the availability of thinner sections on paraffin blocks, enhanced comparison of LM and IF morphology, the capacity to analyze retrospective instances, and the absence of a requirement for cryostat sections.[9]

As a conclusion; We determined that immunofluorescence on paraffin sections can optimally be utilized as a rescue method or to enhance the sensitivity of immunoglobulin detection in instances where immunofluorescence on frozen sections is inadequate. Nevertheless, it cannot supplant standard immunofluorescence in the assessment of kidney biopsy materials.

Data Availability Statement: The data that support the findings of this study are availabe on request from the corresponding author.

Ethics Committee Approval: The study is approved by the Nizam's Institute of Medical Sciences ethics committee with review letter number EC/NIMS/3158/2023 dated 17-06-2023.

Informed Consent: Written informed consent was obtained from the patients who agreed to take part in the study.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – M.S.U.; Design – M.S.U.; Supervision – M.S.U.; Resources – H.A., S.M.A., M.S.U.; Materials – H.A., P.C.; Data Collection and/or Processing – H.A., S.M.A., S.D., M.S.U.; Analysis and/or Interpretation – H.A., S.M.A., M.S.U.; Literature Search – H.A., S.M.A., S.D.; Writing Manuscript – S.M.A., M.S.U.; Critical Review – SM.A., M.S.U., S.B.R., S.G., G.T.

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