

Diagnostic Value of Immunoperoxidase Staining and Immunofluorescence in the Study of Kidney Biopsy Specimens

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Introduction. This study aimed to determine diagnostic value of immunoperoxidase in comparison with immunofluorescence in the diagnostic assessment of kidney biopsy specimens.

Materials and Methods. Forty-eight kidney biopsy specimens were used to compare a direct immunofluorescence technique with immunoperoxidase techniques on paraffin sections. The sensitivity and specificity were calculated. The kappa statistic for agreement between the two tests was categorized as poor (zero to 0.2), moderate (0.21 to 0.45), good (0.46 to 0.75), and almost perfect concordance (0.76 to 1.0).

Results. Compared with immunofluorescence, the immunoperoxidase technique presented a sensitivity of 88.55% and a specificity of 69.22%. Its sensitivity in the staining for IgG, IgM, and IgA was 93.75%, 95.45%, and 76.47%, respectively. The specificity of this test in the staining for IgG, IgM, and IgA was 54.54%, 57.14%, and 96.00%, respectively. The overall kappa value was 0.60 and it was 0.60 for assessing staining intensity. There was a moderate agreement between immunoperoxidase and immunofluorescence in the positive or negative staining for IgG and IgM, as well as a good agreement in the positive or negative staining for IgA. For the staining intensity, the two tests had a good concordance for IgG and IgA and a moderate concordance for IgM.

Conclusions. Although immunoperoxidase method has a lower overall diagnostic performance as compared to immunofluorescence, given the good concordance between the two techniques, it can be an alternative method for immunofluorescence study of kidney biopsy specimens, particularly where immunofluorescence fails or is not available.

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INTRODUCTION

Kidney biopsy is essential for the accurate diagnosis and management of a variety of kidney disorders.¹ Immunofluorescence technique has been commonly applied in various fields of biology for several years, including the evaluation of cells in suspension, cultured cells, tissue, beads, as well as microarrays for the detection of specific proteins.²

The assessment of kidney biopsy specimens by immunofluorescence techniques in addition to light microscopy is extremely helpful to clinicians.³ This tool as a gold standard technique in both research and clinical settings enables scientists and specialists to detect the location of the antibodies which bind to the antigen of interest through conjugation to fluorescent dyes.⁴

Immunofluorescence microscopy is now commonly used to diagnose glomerular diseases. Detection of these diseases requires kidney biopsy and corresponding clinical evidence and biochemical findings.⁴ Immunofluorescence has a major role in this correspondence that can show granular deposition of immunoglobulins and thus confirm the diagnosis and distinguish glomerular diseases from antglomerular basement membrane nephritis.^{5,6} In spite of high diagnostic accuracy of this technique, it may be accompanied with some potential limitations such as photobleaching (due to high intensity of light exposure that can result in photosensitization of singlet oxygen generation by the dye triplet-excited state), autofluorescence in mammalian cells due to flavin coenzymes, and fluorescence overlap of signals.^{7,8}

Immunoperoxidase technique is another tool in which the antibodies are visualized via a peroxidase-catalyzed reaction. In this technique, peroxidase enzyme is used to catalyze a chemical reaction to produce a colored product.⁹ In this diagnostic method, immunostaining is used on tissue biopsies for more detailed histopathological study. Nowadays, traditional immunofluorescence is replaced to a large extent by immunoperoxidase techniques applied to paraffin sections of formaldehyde-fixed tissue.⁹ It seems that the immunoperoxidase method can minimize some practical disadvantages of the immunofluorescence method such as separate tissue specimen and handling, ultraviolet microscopy, fading, and impermanence of the label-making archiving. The diagnostic performance of immunoperoxidase technique in different tissues biopsy has not clearly determined. The present study tried to determine diagnostic value and accuracy of immunoperoxidase in comparison with immunofluorescence in the diagnostic assessment of kidney biopsy.

MATERIALS AND METHODS

Studied Tissue Samples

Forty-eight kidney biopsy specimens from primary and secondary glomerular diseases such as membranous glomerulonephritis, membranoproliferative glomerulonephritis, focal segmental glomerulosclerosis, rapidly progressive glomerulonephritis, minimal change disease, immunoglobulin A (IgA) nephropathy, and lupus nephritis were evaluated. In each sample, fixed

tissue blocks in formalin and embedded in paraffin were extracted. All of the hematoxylin-eosin and immunofluorescent slides were reviewed by pathologists and pathology assistants to verify the recorded diagnosis. From each sample, a paraffin block was selected and chemical immunohistochemistry expression was assessed by a Leica kit (Concord, Canada). We used direct immunofluorescence method on frozen section tissue samples and tissue antigens detected by fluorescein isothiocyanate-labeled antibodies. We also used Dako antibodies and sample protected from light during the staining procedure.

We had 48 specimens, but in a few paraffin blocks, renal glomeruli for immunohistochemistry was not seen due to multiple sections for special stains; therefore, we deleted these blocks and finally we had 43 specimens in this study.

Immunohistochemistry Protocol

The slides were placed in diluted poly-L-lysine solution 1:10 in distilled water for 1 hour. The slides were placed for 24 hours in an autoclave at 37°C until dry. Three-micrometer-thick tissue sections were prepared from selected paraffin blocks and placed on the slides stained with L-lysine. After cutting, the slides were placed at an autoclave at 37°C for 24 hours, and the next day, the slides were placed at 60°C for 1 hour and deparaffinization procedure was performed as follows: 3 times for 5 minutes in xylol, 1 time for 5 minutes in 100% ethanol, 1 time for 5 minutes in 90% ethanol, and 1 time for 5 minutes in 70% ethanol. The slides were washed by distilled water, and then were set into jars containing citrate buffer with a pH of 6 and were boiled for 15 minutes in a microwave. The slides were then run at room temperature. Then the slides were placed in peroxidase blocking solution (3% hydrogen peroxide and methanol) for 10 minutes to neutralize endogenous peroxidase and then washed in distilled water for 5 minutes. The slides were washed at phosphate buffered saline for 10 minutes. One drop of yellow vial peroxidase was shed on each weave and it remained on it for 5 minutes. The slides were washed in phosphate buffered saline on a rotator for 5 minutes. A drop of the block Novolink protein solution was shed on the tissue and after 5 minutes, tissue was washed with phosphate buffered saline for 10 minutes. The slides were incubated with proteinase K for 5 minutes and

then were washed at phosphate buffered saline for 5 minutes on a rotator. Primary antibody was then added and after an hour was washed by phosphate buffered saline for 10 minutes. At this stage, the post primary Novolink (red) was added, and after 30 minutes, it was washed by phosphate buffered saline. A drop of polymer Novolink (purple) was added, and after 30 minutes, it was washed by phosphate buffered saline. Finally, 950 lambda of 3,3'-diaminobenzidine buffer substrate and 50 lambda of 3,3'-diaminobenzidine chromogen, ready 1 hour before, were added to the tissue (15 to 10 minutes) and then was washed by distilled water. For the background color (counter stain), the tissues were stained by hematoxylin for 30 seconds. The slides were washed and dehydrated (alcohol-xylol).

A sample of tonsil tissue as a positive control and a sample of tissue from the wall of the gallbladder as a negative control were used for markers of IgG, IgM, and IgA. Then, the strained samples were evaluated by light microscopy by a pathologist and a pathology assistant in terms of staining and its intensity. Staining for IgG, IgA, and IgM were recorded as positive or negative for immunofluorescence and immunoperoxidase. For immunoperoxidase, the overall (average) staining intensity was given a value of zero (no stain), 1 (1% to 25% stain), 2 (26% to 50% stain), or 3 (> 50%), and for immunofluorescence staining, the staining intensity was classified as negative, weak, or strong based on the laser power and detector gain settings used for image acquisition in combination with the visual appearance of the image.

Statistical Analysis

Results were presented as mean ± standard deviation for quantitative variables and were summarized as absolute frequencies and percentages for categorical variables. Correlation between the quantitative variables was examined using the Pearson correlation coefficient test. The diagnostic performance of immunoperoxidase according to the results of immunofluorescence as a gold standard test was measured by the quantity of the true and false positive and negative results. The sensitivity and specificity were calculated. The kappa statistic was used to measure agreement between the two tests and kappa values from zero to 0.2 indicated a poor correlation; from 0.21 to 0.45, moderate;

from 0.46 to 0.75, good; and from 0.76 to 1.0, almost perfect. For the statistical analysis, the SPSS software (Statistical Package for the Social Sciences, version 19.0, SPSS Inc, Chicago, Ill, USA) was used. *P* values less than .05 were considered significant.

RESULTS

Compared with immunofluorescence, the immunoperoxidase technique presented a good concordance rate yielding a sensitivity of 88.55% and a specificity of 69.22% (Table). The sensitivity of the immunoperoxidase in the staining for IgG, IgM, and IgA was 93.75%, 95.45%, and 76.47%, respectively. Meanwhile, the specificity of this test in the staining for IgG, IgM, and IgA was 54.54%, 57.14%, and 96.00%, respectively (Figures 1 to 3). The overall kappa value was 0.60 and it was

Positive and Negative Immunoperoxidase Staining in Relation to Immunofluorescence in Pathological Examinations of Kidney Biopsy Specimens

Immunofluorescence	Immunoperoxidase		Total
	Negative	positive	
Staining for IgG			
Negative	6	5	11
Positive	2	30	32
Total	8	35	43
Staining for IgM			
Negative	12	9	21
Positive	1	21	22
Total	13	30	43
Staining for IgA			
Negative	24	1	25
Positive	4	13	17
Total	28	14	42

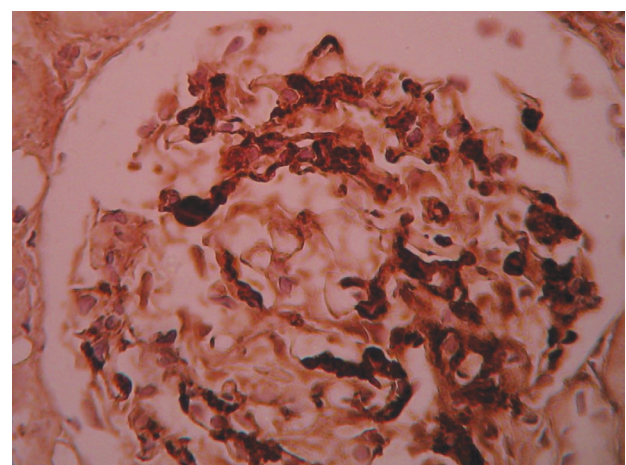


Figure 1. The IgA staining in a patient with IgA nephropathy (× 400).

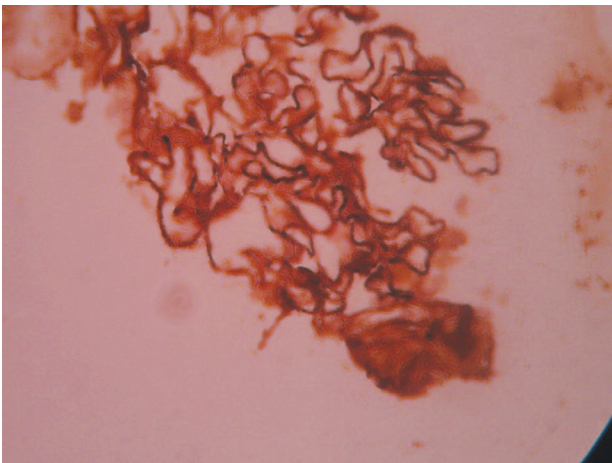


Figure 2. The IgG staining in a patient with membranous nephropathy ($\times 400$).

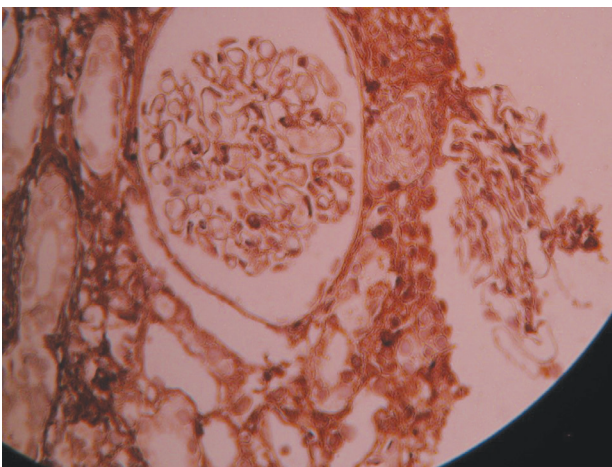


Figure 3. No staining of markers in a patient with minimal change diseases ($\times 400$).

0.60 for assessing staining intensity. There was a moderate agreement between immunoperoxidase and immunofluorescence in the positive or negative staining for IgG and IgM, as well as a good agreement in the positive or negative staining for IgA, with the kappa values of 0.53, 0.53, and 0.75, respectively. Moreover, in the staining intensity, the two tests had a good concordance for IgG and IgA (kappa, 0.63 and 0.73, respectively) and a moderate concordance for IgM (kappa, 0.42).

DISCUSSION

Comparing the diagnostic value of immunoperoxidase against the immunofluorescence method, our study showed different sensitivity and specificity values in the staining for different types of antibodies. We revealed a high sensitivity in the staining for IgG and IgM, but high specificity in the

staining for IgA. Meanwhile, the two techniques had moderate to good agreement in the staining presence and intensity. In a similar study by Mölne and colleagues, concordant observations between the two techniques were 71% for all, 82% for IgG, and 89% for IgA.¹⁰ In another study by Kemény and colleagues evaluating different immunohistological methods on 30 kidney biopsy cases, the direct immunoperoxidase method gave identical results with immunofluorescence in 88% concerning IgG.¹¹ Howie and coworkers also found an agreement between immunoperoxidase and immunofluorescence in the staining for IgG, IgA, and IgM in 50 biopsy specimens and discordant findings did not affect the diagnosis.¹²

Immunofluorescence on frozen tissue is the method of choice with a high sensitivity for the study of renal diseases so that in the diagnosis of some kidney diseases such as lupus nephritis, acute postinfectious glomerulonephritis, cryoglobulinemic glomerulonephritis, fibrillary glomerulonephritis, primary amyloidosis, myeloma cast nephropathy, and light-chain Fanconi syndrome, with a sensitivity of 100%. In other types of kidney diseases, its sensitivity ranged widely between 20% (for antiglomerular basement membrane disease) and 88% (for immunoglobulin IgA nephropathy).¹³ However, the immunoperoxidase method has been shown to have an overall lower sensitivity for the detection of complement C3 and IgG in different kidney disease categories.¹³

Regarding concordance between immunoperoxidase and immunofluorescence in the staining for IgG, IgA, and IgM in kidney biopsies, we obtained an acceptable concordance, especially in the staining for IgA. In Sinclair and associates' study, immunoperoxidase staining of paraffin sections was at least as reliable and sensitive as direct immunofluorescence on fresh tissue in detecting immune deposits in kidney biopsies.¹⁴ In their study, overall concordance rate was 79%, similar that reported by McIver and colleagues.¹⁵ Therefore, the highest agreement between the two techniques can be specified in the staining for IgA. However, as a limitation of our study, some biopsy specimens did not have glomerulus, and therefore were excluded from the study.

CONCLUSIONS

Although immunoperoxidase method is not

superior to immunofluorescence because of its lower overall diagnostic performance, regarding the obtained good concordance between the two techniques, it can be an alternative method for kidney biopsies, particularly in unavailability of immunofluorescence or the presence of its disadvantages.

CONFLICT OF INTEREST

None declared.

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