DIRECT IMMUNOFLUORESCENCE AND IMMUNOHISTOCHEMISTRY IN DIAGNOSTICS OF GLOMERULONEPHRITIS

Zinaida Karasalihović[1*], Ermina Iljazović[1], Dušan Ferluga[2], Elmir Čičkušić[1], Jasminka Mustedanagić Mujanović[1], Jugoslav Stahov[3], Ina Škaljić[1]

[1] Polyclinic for Laboratory Diagnostic University Clinical Centre Tuzla, Trnovac 1, 75000 Tuzla, Bosnia and Herzegovina

2 Institute of Pathology, Faculty of Medicine, University of Ljubljana, Korytkova 2, 1000 Ljubljana, Slovenia

3 Faculty of Natural Sciences and Mathematics, University of Tuzla, Univerzitetska 1, 75000 Tuzla, Bosnia and Herzegovina

* Corresponding author

Abstract

The needle biopsies from 60 transplanted and native kidneys have been processed and a prospective analysis of pattern, intensity and distribution of immunoglobulin deposits (IgA, IgG and IgM) and complement components (C3c and C1q) identified in these lesions has been carried out by immunohistochemistry with three step immunoperoxidase, in the period from 2000 to 2004. Those deposits were previously detected and analyzed by immunofluorescence. The samples consisted of 30 renal biopsies, previously diagnosed with glomerulonephritis and positive immunofluorescence and 30 renal biopsies without morphologic changes and deposits on immunofluorescence. 78,7% of the analyzed samples showed the identical results of the deposits of immunoglobulin and components of the complement with both, immunohistochemistry and immunofluorescence method. Sensitivity of the immunohistochemistry method with three step immunoperoxidase for all analyzed immunoglobulin and complement components is high (0,93), while specificity for the same method is 0,79. Standardized method of the three step immunoperoxidase on the paraffin embedded, formalin fixed needle renal biopsies could successfully replace the immunofluorescence method in diagnostic of GN, with the emphasis on a follow up and control of each single step in the procedure of the method.

KEY WORDS: immunofluorescence (IF), immunoperoxidase (IP), renal biopsies, immune deposits

Introduction

The approaches to morphologic analysis of renal biopsies are limited, as is the number of pathognomic morphological changes. One of the key elements for diagnosis of glomerulonephritis is the finding of immunoglobulin deposits and deposits of complement components exclusively analyzed by the method of direct immunofluorescence (DIF), which has been a gold standard for diagnosis of immunological lesions on renal biopsies for half a century (1). Even though it is technically relatively simple laboratory method, it is not an easy task to perform with high quality results. This method is also highly sensitive and specific, although the fluorescence is not permanent, and its documentation is only possible with quality microphotography, which is not always available. For immunofluorescence analysis, in notable number of cases "locating" fluorescent immune deposits in the context of morphological picture is complicated, due to hardly noticeable signal. The reasons mentioned above led to development and use of immunohistochemistry method on paraffin tissue sections, instead of DIF method (2,3). If immunohistochemistry method would allow satisfactory view of immune deposits, a form of microscopic diagnostics in which immune deposits could be viewed in context of clearly discernable and quality background, would be provided. At the same time, stable slides would be secured, for further analysis of immune deposits (4). The objective of our work was to analyze distribution, pattern and intensity of immunoglobulin (IgA, IgG, IgM) and complement components (C3c and C1q) on renal biopsies using immunohistochemistry method of three step immunoperoxidase, and compare sensitivity of this method to the method of direct immunofluorescence.

Materials and Methods

Renal tissue samples

The paper represents retrospective-prospective study which involves detection and analysis of distribution, pattern and intensity of immunoglobulin deposits (IgG, IgA, IgM) and complement components (C3c and C1q) by immunohistochemistry method of a three step immunoperoxidase in the 60 renal biopsies from the archive of Department of Pathology, Polyclinic for laboratory diagnostics of University Clinical Center in Tuzla, in the period from 2000 to 2004. Previously performed detection of immune deposits done by immunofluorescence method on the same biopsy samples represents control group. Out of 60 examined, 30 biopsies were chosen by the random selection method from the pool of 97 biopsies, with previously diagnosed glomerulonephritis and confirmed positive result deposits of immunoglobulin and complement components by immunofluorescence method. A second group includes 30 biopsies without apparent glomerular morphological changes and with the absence of immunoglobulin and complement components deposits analyzed by immunofluorescence method. All of the biopsy samples were adequately fixed and contained enough renal tissue for additional analysis.

IF Technique

All of the tissue samples have been transported to Department of Pathology in physiological solution, according to standard operating procedure for renal biopsies and treated with method of cold fixation upon arrival. Antibodies labeled with fluorescein-isothiocyanate (FITC) against human immunoglobulin (IgA, IgG, IgM) and complement components (C3c and C1q) were applied on histology sections 4μm thick (Table1). All antibodies are manufactured by DAKO, Glostrup, Denmark. Slides are analyzed by microscope Diastar Reichert-Jung with an appropriate filter for FITC fluorescence. A pattern and distribution of immune deposits were analyzed qualitatively, while the intensity was determined semi-quantitatively by 4 degree scale from 0 to 3+, as follows: complete absence of deposit (0), mild (1+), moderate (2+), strong (3+) intensity. Upon completion of DIF analysis tissue cylinders were fixated in 4% buffered formaldehyde (pH 7,2-7,4), paraffin embedded, and histology sections 4μm thick were stained with hematoxylin eosin method for the analysis of basic light microscopic morphological marks and following special staining; Periodic acid-Schiff reagents (PAS), Trichrom-Masson, Van Gieson-Weigert, Impregnation with silver according to Jones. Samples are analyzed with Diastar Reichert-Jung light microscope with the goal of determining the type and stage of morphologic changes of all of the components of renal tissue. Classification was done according to the criteria of WHO for glomerular diseases and Banff 97 work classification for renal transplantation pathology (5,6).

Detected antigen	Clone	Antibody (mg/l)	Dilution
IgA	Polyclonal	200	1:50
IgG	Polyclonal	400	1:50
IgM	Polyclonal	400	1:50
C3c	Polyclonal	400	1:50
C1q	Polyclonal	1100*	1:30

* Total protein concentration

TABLE 1. Antibodies used for DIF

IP Technique

Paraffin blocks of the same tissue cylinders are treated according to protocol for immunohistochemistry method of three step immunoperoxidase with streptavidine (IP). Histology sections 4μm thick were placed on organosilan pretreated slides and incubated over night on 37°C. Slides were deparaffinized in xylol-ethanol at room temperature and incubated 30 minutes in 1,5% H_2O_2 in methanol because of blocking of the endogenous activity of peroxidase. Retrieval of antigens with the process of proteolytic enzyme digestion was performed with pretreatment of slides in Pronase (Roche, Manheim, Germany, 0,01g-0,02g/100ml Sodium dodecyl sulfate, pH =7,8) at 37°C in duration of 6 minutes, with the exclusion of slides with determined presence of IgM deposits, pretreated in citrate buffer (10 mmol/dm3, pH=6,0) at 100°C in duration of 10 minutes. Next step is treatment of histology slides with avidine-biotin blocking solution in duration of 10 minutes, two times at room temperature (Dako Cytomation, Carpinteria, USA). Histology slides were mounted on appropriate carriers and placed in center for immune-staining Sequenza-Shandon where all stages of incubation were performed. Pre-incubation stage of 15 minutes with 10% normal fetal calf serum is followed with procedure of three step immunoperoxidase. In the first stage slides are incubated with primary antibodies in duration of 60 minutes. Following antibodies were used: IgA, IgG, IgM, C3c and C1q, all manufactured by DAKO, Glostrup, Denmark (Table 2). In the second stage incubation in duration of 30 minutes is performed with biotin labeled anti-rabbit antibody. Incubation in the third stage is done with streptavidine labeled peroxidase in duration of 30 minutes. Washes between incubation are done with phosphate buffered saline. Peroxidase activity is developed with 3,3 diaminobenzidine tetrachloride solution and H_2O_2 substrate. Contrast staining is done with hematoxylin, and upon dehydratation slides were mounted with Canadian balsam. Evaluation of technical quality of slides treated with immunohistochemistry method of three step immunoperoxidase is performed by light microscope. The quality of specimen is evaluated as unsatisfactory in cases with present non specific positive staining of adhering serum proteins in glomerular and peritubular capillaries, consequence of poor proteolysis and indirectly an indication of inadequate antigen retrieval (2,7). Samples observed under light microscope exhibiting tissue damage in pattern of dissolution of cytoplasm and/or cell nuclei as a consequence of an excessive digestion are also evaluated as samples of inadequate quality. In both cases analysis has been repeated on the new sections of the same tissue samples. Immunoglobulin and complement components deposits were analyzed under light microscope Olympus BX40, qualitatively and semi-quantitatively, according to the same module that is used in the method of direct immunofluorescence (distribution, pattern and intensity), and results are compared to written description of findings performed by immunofluorescence method.

Detected antigen	Clone	Antibody (mg/l)	Dilution §
IgA	polyclonal	2200	1:15000
IgG	polyclonal	5900*	1:15000
IgM	polyclonal	6000*	1:15000
C3c	polyclonal	7000	1:20000
C1q	polyclonal	3700*	1:20000

* Total protein concentration
§ Buffered saline solution, pH 7,8

TABLE 2. Antibodies used for IP

Statistical analysis

All analyzed samples are included in 2x2 table of paired samples in which immunofluorescence method was regarded as the reference test. Statistical analysis has been calculated with the continuity correction included and referred to the chi-square distribution for probability with 1df (McNamara test).

RESULTS

Comparison of immunofluorescence and immunohistochemistry method

IgA

In the 47 (78,3%) biopsy samples of the examined group, finding of IgA deposit by IP was identical to DIF finding according to intensity, distribution and pattern of deposits (Figure 1). In 5 (8,3%) cases intensity of deposits found by IP was more expressed (Figure 5), in 1 (1,7%) case DIF found deposits were of stronger intensity, while the pattern and distribution of deposits inside glomerules were identical . In 6 (10%) cases presence of IgA deposit in biopsy samples was observed by IP with a negative immunofluorescence finding, while 1 (1,7%) case had a positive immunofluorescence finding deposits of this immunoglobulin were not observed by IP. Statistical analysis has shown high specificity (0,93) and sensitivity (0,88) of IP method. Predicted value of positive test for IP method was 0,93. Statistically significant difference was not found in predictions of these two methods (Chi=0,16, P=0,68).

IgG

In total of 49 (81,6%) renal biopsies finding of IgG depos-

it by immunohistochemistry method of three stage immunoperoxidase was identical to immunofluorescence finding according to intensity, distribution and pattern of deposit. In 3 (5%) cases IP method showed deposits were more expressed according to intensity, while in 1 (1,7%) case DIF deposits were of stronger intensity, with identical distribution inside glomerules and pattern of deposit. In 4 (6,7%) cases IP method showed presence of IgG deposit in biopsy samples with negative DIF finding, and in 3 (5%) cases with positive DIF finding, IP deposits of this immunoglobulin were not observed. Statistically significant difference was not found in predictions of these two methods (Chi=0, P=1). Statistical analysis showed high specificity (0,87) and sensitivity (0,90) of immunohistochemistry method. Predictive value of positive test for immunohistochemistry method was 0,87.

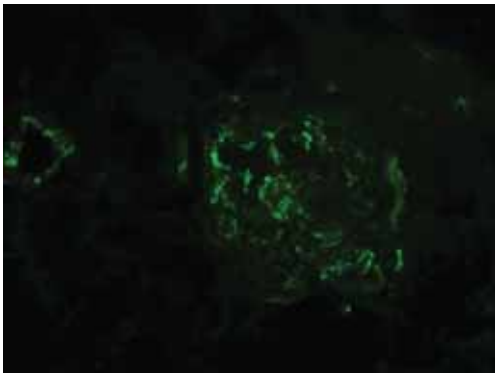

FIGURE 1. IgA granular deposits intensity (Immunofluorescence, 400x)

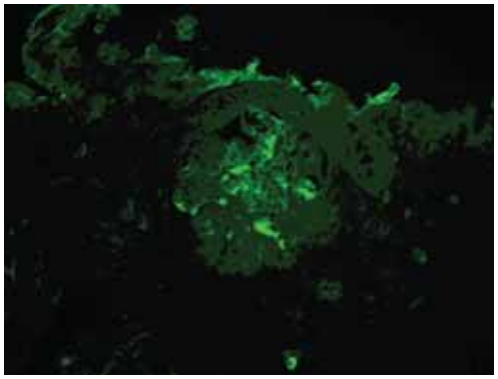

FIGURE 2. IgM focal granular deposits (Immunofluorescence, 400x)

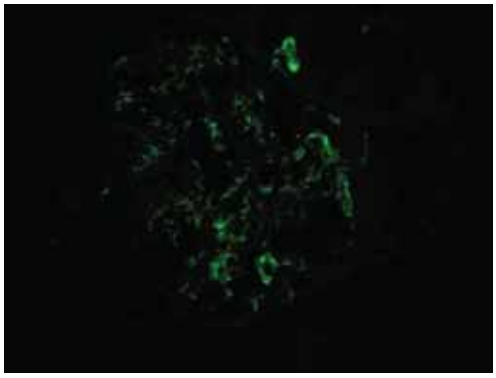

FIGURE 3. C1q granular and "smudgy" deposits (Immunofluorescence, 400x)

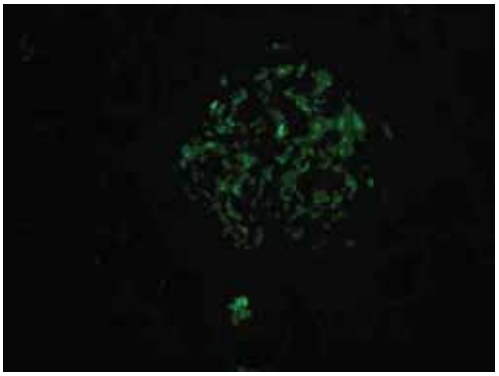

FIGURE 4. C3c granular and linear deposits (Immunofluorescence, 400x)

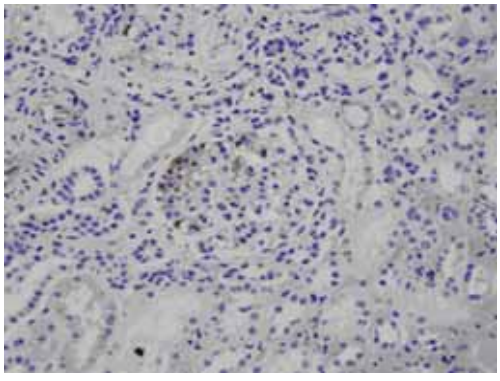

FIGURE 5. IgA focal granular deposits (Immunoperoxidase, 400x)

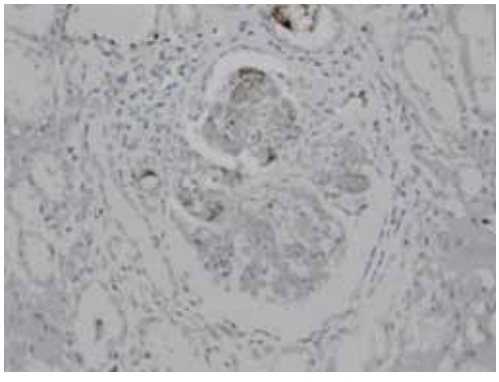

FIGURE 6. IgM focal granular deposits (Immunoperoxidase, 400x)

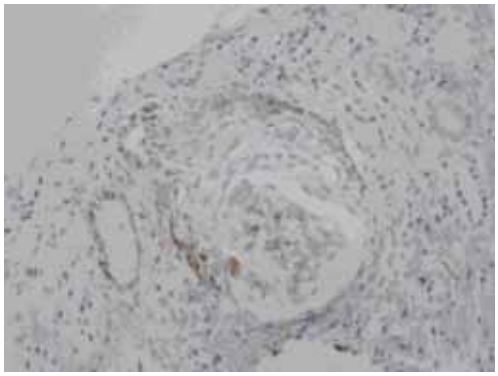

FIGURE 7. C1q focal granular deposits (Immunoperoxidase, 400x)

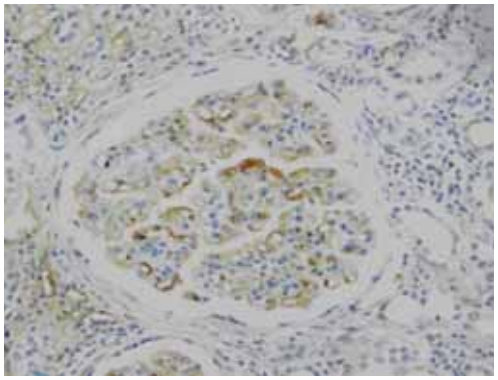

FIGURE 8. C3c granular deposits (Immunoperoxidase, 400x)

IgM

In total of 49 (81,6%) renal biopsies finding of IgM deposit by immunohistochemistry method of three stage immunoperoxidase was identical to finding of immunofluorescence method according to intensity, distribution and pattern of deposit (Figures 2,6). Immunohistochemistry evidence of deposits was of stronger intensity in 3 (5%) cases, while in 2 (3,3%) samples DIF method detected deposits of stronger intensity. In 6 (10%) cases IP detected IgM deposits on samples with previous negative DIF finding. Statistically significant difference in predictions of DIF and method of three step immunoperoxidase was not found (Chi=4,17, P=0,412). Statistical analysis indicates high specificity (0,81) and sensitivity (1) of IP method. Predictive value of positive test for immunohistochemistry method is 0,82.

C3c

In 51 (85%) analyzed biopsies, finding of C3c deposit and complement components, finding of IP method was identical to DIF finding according to intensity, pattern and distribution of deposits (Figures 4,8). In 1 (1,7%) case intensity of deposits detected by IP had slightly stronger expressed. In 7 (11,6%) cases IP method demonstrated presence of C3c in biopsies which had negative DIF finding, and 1 (1,7%) case with positive DIF finding IP method did not detect deposit of this complement component. Statistically significant difference was not found in predictions of these two methods (Chi=3,125, P=0,077). High sensitivity (0,96) and specificity (0,80) of method of three step immunoperoxidase was determined with statistical analysis. Predictive value of positive test for this method is 0,77.

C1q

In 40 (65,7%) biopsy samples of both groups, immunohistochemistry finding of deposit C1q complement component was identical to finding of DIF (Figure 3), and in all cases with present deposits they had identical intensity, pattern and distribution. In 20 (33,3%) cases analyzed by IP method presence of deposit C1q was found in biopsy in which DIF finding was negative. All detected deposits in these biopsies had intensity of 1+ and granular shape localized in the wall of glomerular capillaries (Figure 7). In predictions for the two compared methods statistically significant difference (Chi=15,05, P=0,0001) has been found. The evidence is high sensitivity (1) and low specificity (0,64) of immunohistochemistry method of three step immunoperoxidase. Predictive value of a positive test for immunohistochemistry method is 0,17.

In general

A comparison of the results for all analyzed immunoglobulin and complement components established that in 236 (78,7%) samples findings were identical for both methods. In 17 (5,6%) renal biopsies with positive finding with both methods, deposits of various intensities are detected. In 41 (13,7%) tissue sample with previously negative DIF finding IP method detected deposits of all immunoglobulin and complement components. In 6 (2%) cases IP did not confirm presence of deposits in biopsy samples with previously positive immunofluorescence.

Antibodies	Method	Negative	Positive	
			Intensity 1+	Intensity 2 +
IgA	DIF	32	25	3
	IP	26	24	10
IgG	DIF	31	27	2
	IP	30	26	4
IgM	DIF	33	25	2
	IP	26	31	3
C3c	DIF	35	25	0
	IP	29	30	1
C1q	DIF	56	4	0
	IP	36	22	2

TABLE 3. Presence and intensity of immunoglobulin and complement components deposits

DIF/IP	(+/+)	(-/+)	(+/-)	(-/-)	Number	Sensitivity	Specificity
IgA	27	6	1	26	60	0,88	0,93
IgG	26	4	3	27	60	0,9	0,87
IgM	28	6	0	26	60	1	0,81
C3c	24	7	1	28	60	0,96	0,8
C1q	4	20	0	36	60	1	0,64
Σ	**109**	**43**	**5**	**143**	**300**	**0.93**	**0,79**

TABLE 4. Results of immunofluorescence and immunohistochemistry method

Results acquired by comparison of the two methods for all analyzed antibodies are shown in Tables 3 and 4. Statistically significant difference was found in prediction of immunofluorescence method and method of three step immunoperoxidase (Chi=20,45, P<0,0001). Statistical analysis showed high sensitivity (0,93) of immunohistochemistry method, while specificity is 0,79. Predictive value of a positive test for this method is 0,74.

Discussion

According to our knowledge and Molne report, only four studies, which compared immunofluorescence and immunohistochemistry method results of immune deposits detection in renal biopsies in a way to allow statistical comparison with our results, have been reported. (8) In three studies from earlier periods the authors compared results of DIF-e with immunohistochemistry method peroxidase-antiperoxidase, while Molne in his work used method of immunoperoxidase with Dako En Vision HRP system (8,9,10,11). Considering that basic postulate for acquiring quality slides by immunohistochemistry method are standardized procedure and educated and experienced laboratory personnel, it was decided for the method of three step immunoperoxidase with streptavidine, which has been used in our lab in different tissue samples over one decade. This will possibly lead to eventual introduction of this method as a routine diagnostic procedure of renal disease in the future. Identical results acquired with comparison of DIF and immunohistochemistry method which in studies of MacIver, Sinclair and Howie is from 82% do 88% is higher from the value we obtained (78,7%), while the value from the study done by Molne and associates is somewhat lower (70,9%) (8,9,10,11). Discrepancy between these results done by immunofluorescence and immunohistochemistry method in our and studies mentioned above is a consequence of distinction of application of criteria for determining uniformity of positive findings. We considered identical only findings in which deposits were of same intensity, while the same criteria was not taken in to consideration in compared studies. Statistical analysis in total results of the studies done by McIver and Sinclair did not found significant difference between DIF and IP in detection of immunoglobulin and complement components (9,10). In studies done by Howie, Molne and in our study significant difference between these two methods has been observed (p<0,001) (8,11). Looking independently at the results of individual immunoglobulin and complement components in both mentioned studies for IgA and IgG significant difference was not found, although our study found noticeable and statistically significant difference only in results of C1q complement components. This significant difference, looked at in whole is a consequence of found differences in analysis of individual antibodies in the two compared methods. In the study done by Howie and associates C3c is more often detected with fluorescent method, while in Molne's study C1q and IgM are more often detected by immunohistochemistry method (8,11). In our study, all analyzed immunoglobulin and complement components are detected in certain number of renal biopsies with previously negative immunofluorescence finding. In the study done by Turner and associates in 1979 comparison of deposit findings for IgA, IgG, IgM and C3c by immunofluorescence and immunoperoxidase method in 200 of renal biopsies expressed uniformity in 81% to 90% cases (12). Difference in relation to percentage of identical findings in our study is caused by the fact that in mentioned study C1q complement component was not analyzed, while our study reported decrease in percentage of identical findings (65,7%). In the study done by Jackson and associates in 1990, in 69 renal biopsies immunofluorescence method is compared with immunohistochemistry method of immunoalkaline phosphatase, with the difference that in our study alkaline phosphatase is used as a substrate for deposit labeling (13). Uniformity of 85% for all compared antibodies (IgA, IgG, IgM, C3c, C1q and fibrinogen) is different from our results, probably a consequence of the difference in compared antibodies, due to the fact that in our study we did not analyze presence of fibrinogen deposits. In the reference to the role of complement activation in etiopathogenesis of glomerulonephritis, deposits of complement components have a diagnostic role, especially in determining glomerular damage in the context of systematic diseases. In our study in 11,6% cases we detected C3c deposits in biopsies with previously negative

immunofluorescence finding, differing from the results of Jackson and associates, where weak correlation with immunofluorescence finding was caused with increased number of immunohistochemistry negative/DIF positive findings (13). This difference in results could be explained with the use of different pretreatments. In our study significant difference is found in detection of C1q complement component, which is by IP method detected in 20 renal biopsies with previously negative DIF finding. Large number of DIF negative/IP positive results could compromise specificity of used antibody for this antigen in fixed paraffin samples. According to the opinion of Molne (8), it is more probable that during tissue preparation for immunofluorescence some factors are relocated or removed, which disables their retrieval and detection with this method. On the other hand, IP technique allows detection of antigens lost in the process of tissue preparation for immunofluorescence analysis, especially in the process of tissue washes. According to the research done on immunohistochemistry staining of renal tissue, especially for observation of immunoglobulin and complement components in deposits of immune complexes, the most effective pretreatment is the one with proteolytic digestion (9). Duration of digestion must be adjusted to the stage of fixation and thickness of histology sections which need to be constant. In our study sections paraffin embedded tissues previously fixed in 4% formaldehyde buffer were used. Duration of fixation process also needs to be standardized, depending on specimen dimensions, because it enables fast and uniform process of fixation, as a precondition for controlled antigen retrieval.

Conclusion

Three step method of immunoperoxidase is sensitive and specific in determining deposits of immunoglobulin and complement components in renal biopsies, and it has certain advantages when compared to immunofluorescence method (2). Considering different results, in the use of this method, authors recommend parallel use of immunofluorescence and immunoperoxidase method as a way of results verification. According to the results of our study in the cases when the archive material was accessible and previous process of fixation standardized, retrospectively prospective study can exclude need for long lasting parallel use of two methods.

Standardized method of three step immunoperoxidase can represent progress toward elimination of immunofluorescence as a most significant method in detection of immune complex in renal biopsies. Each procedural step, including starting preparation of tissue sample, has to be controlled and standardized, for the purpose of getting reliable results. Excellent technical advancement in immunohistochemistry methods, wide use in all areas of pathology and well trained laboratory personnel will enable use of three step immunoperoxidase as an equivalent method in analysis of immune deposits in renal biopsies, especially in case when fresh tissue is not available for immunofluorescence.

List of Abbreviations:

Ig	-	Immunoglobulin
IF	-	Immunofluorescence
DIF	-	Direct immunofluorescence
IP	-	Immunoperoxidase
FITC	-	Fluorescein-isothiocyanate
PAS	-	Periodic acid-Schiff
WHO	-	World Health Organization

References

(1) Kashagarian M., Immune complex In: Atlas of diagnostic immunohistopathology (True L.D., editor). Lippincot, Philadelphia, 1990; pp.: 1711-1735.

(2) Furness P.N., Boyd S. Electron microscopy and immunocytochemistry in the assessment on renal biopsy specimens: actual and optimal practice. J. Pathol. 1996; 49:233-237

(3) Howat A.J., Thomas C.M., Coward R.A. Immunoperoxidase for the demonstration of immune deposits in renal biopsies. Curr. Diagn. Pathol. 2000; 6:125-129

(4) McIver A.G., Mepham B.L. Immunoperoxidase techniques in human renal biopsy. Histopathology.1982; 6 (3): 249-267

(5) Churg J., Bernstein J., Glassock R.J. Renal disease: Classification and atlas of glomerular diseases. 2nd ed. Igaku-Shoin, Tokyo, 1995. pp.: 4-17.

(6) Solez K., Axelsen R.A., Benediktson H., Burdick J.F., Cohen A.H., Colvin R.B., et all. International standardization of criteria for the histology diagnosis of renal allograft rejection. Kidney Int. 1993; 44: 411-422.

(7) Thomas C.M., Howat A.J. Assessment of renal biopsy specimens. Clin. Pathol.1996; 49:233-237.

(8) Molne J., Breimer M.E., Svalander C.T. Immunoperoxidase versus immunofluorescence in the assessment of human renal biopsies. Am. J. Kidney Dis. 2005; 45:674-683.

(9) McIver A., Giddings J., Mepham B. Demonstration of extracellular immunoproteins in formalin-fixed renal biopsy specimens. Kidney Int. 1979;16:632-636.

(10) Sinclair R.A., Burns J., Dunnil M.S. Immunoperoxidase staining of formalin-fixed, paraffin-embedded, human renal biopsies, with a comparison of the peroxidase- antiperoxidase (PAP) and indirect methods. J. Clin. Pathol. 1981; 34:859 - 865.

(11) Howie A.J, Gregory J., Thompson R.A., Adkins M.A., Niblett A.J. Technical improvements in the immunoperoxidase study of renal biopsy specimens. J. Clin. Pathol. 1990; 43:257-259

(12) Turner D.R., Wilson D.M., Lake A., Heaton J.M., Leibowitz S., Cameron J.S. An evaluation of the immunoperoxidase technique in renal biopsy diagnosis. Clin. Nephrol. 1979; 11:13-17.

(13) Jackson R., Holme E.R., Phimister G.M., Kennedy A., McLay A.L.C. Immunoalkaline phosphatase technique applied to paraffin wax embedded tissue in diagnostic renal pathology. J. Clin. Pathol. 1990; 43:665-670.