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A Comparative Study of Quality of Antigen Retrieval for Immunohis to Chemistry by Clipping of Slides and Conventional Method

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ABSTRACT
Immunohistochemistry (IHC) is an effective technique focused on microscopy for visualizing cellular components in tissue samples, such as proteins or other macromolecules. At most places, antigen retrieval is done by conventional non clipping method which has its effects on antigen retrieval and quality of immunostaining. Keeping it in view our study planned to compare the quality of antigen retrieval for Immunohistochemistry by clipping of slides and conventional method (Non clipping method) andto access the quality of IHC staining by clipping of slides and by conventional pressure cooker method. For the purpose of the study, a total of 50 sections were taken, 25each from skin and lymph node formalin fixed paraffin embedded sections. The antigen retrieval was determined by both the conventional method (Non-clipping) and the clipping of slides. It was concluded that the slides which are clipped have less chances of damage or destruction as compared to the non-clipped. Also, the tissue exhibits good immunostaining by clipping method as compare to non-clipping method.
Keywords: Immunohistochemistry (IHC), Antigen retrieval, immunostaining, skin biopsy,Lymphnode biopsy,Conventional Pressure cooker

I. INTRODUCTION

Immunohistochemistry (IHC) is an effective technique focused on microscopy for visualizing cellular components in tissue samples, such as proteins or other macromolecules. In the form of different cell types, biological states, and/or subcellular localization inside the complex form of tissues, the power of the IHC is the inborn visual production that can reveals the localization and presence of the target protein.

Technology:

Tissue preparation:

In IHC, the tissue plays a central role and it is necessary to process it so that we can preserve the epitopes and the correct morphology. The preparation of formalin-fixed paraffin-embedded (FFPE) tissue blocks is the most common treatment for IHC. Formalin fixation chemically creates protein cross-linking inside the tissue which results in end of all cellular processes and the cellular components gets freezed at that place and in the conformation, they were in at the time of fixation. It also prevents degradation also. After fixation the tissue is further processed and finally embedded in paraffin blocks. These are then sectioned using a microtome into thin slices (usually 4-5μm). Before further processing, the tissue parts are placed on to glass slides and allowed to adhere.

Antigen (Epitope) retrieval: Antigen retrieval methods

While fixation is important for tissue morphology preservation, this process may also have a negative impact on the detection of IHC. Fixation can change the biochemistry of proteins so that it hides the epitope of interest and can no longer bind to the primary antibody. Epitope masking can be triggered by cross-linking of amino acids within the epitope, cross-linking of unrelated peptides at or near the epitope, altering the epitope's conformation, or altering the antigen's electrostatic charge. Retrieval of antigen refers to any procedure in which an epitope's masking is reversed and epitope-antibody binding is recovered.

Antigen retrieval techniques:

There are different strategies to recover an epitope's immune reactivity. Methods as simple as altering the pH or cation concentration of the diluent of the antibody can affect an antibody's affinity for its epitope.

Techniques are categorized into two major groups- Protease-induced epitope retrieval (PIER) and Heat-induced epitope retrieval (HIER) when addressing antigen retrieval methods.

Protease-induced Epitope Retrieval (PIER):

Enzymes including Proteinase K, trypsin, and pepsin have been used successfully in the PIER technique to restore an antibody's attachment to its epitope. It is thought that the mechanism of action is the peptides cleavage that may mask the epitope. The drawbacks of PIER are the low rate of success in restoring immunoreactivity and the propensity for both tissue morphology and antigen of interest to be destroyed.

Heat-induced Epitope Retrieval (HIER):

Heat induced epitope retrieval has much more promising results in antigen retrieval in general than protein induced epitope retrieval. Vegetable steamer, Microwave ovens, pressure cookers, autoclaves, or water baths are used to conduct HIER.

Antibody binding:

For any IHC dependent technique, the consistency and specificity of the binding molecule is important, and the choice of binding agent will directly affect the result, reliability, and may also affect the interpretation of assay.

Two major types of antibodies are used:

(1). Polyclonal antibodies (a diverse mixture of antibodies that attach the target to various epitopes)

(2). Monoclonal antibodies (that bind the same epitope to everything).

Because of its nature to detect and bind multiple epitopes on the same target, polyclonal antibodies are considered to be more potent. The epitopes on which polyclonal antibodies bind they are usually poorly described and there is increased risk of off-target binding events and background noise also comes with multiple and varying epitope-specificities.

Antibodies, peptides, antibody fragments or other small molecules are the different types of binding molecules can be used in IHC based techniques.[(1)]

Detection systems:

Using a secondary antibody that carries a pre-bound reporter molecule i.e. an enzyme or fluorophore, is the most common way to add a detection device to the experiment. Secondary antibodies are typically specifically aimed at molecules of antibodies from various types of animals.[(2)]

The most popular detection technique for FFPE tissue samples is to use enzymatic reactions to produce a colored precipitate at the site of antibodies binding. Secondary antibodies then bring an enzyme, e.g. horseradish peroxidase (HRP) or alkaline phosphatase (AP), which can transform chromogens such as 3,3' diaminobenzidine (DAB) into brown or bluish precipitates deposited at the reaction site in the tissue.

Counterstaining:

During IHC staining after chromogens application when hematoxylin is used it leads to contrast enhancement and facilitation of observation of histological features. Hematoxylin stains cell cytoplasm with a pale bluish color, and stain cell nuclei in a darker bluish nuance.It is the most common type of counterstain used for FFPE samples. The only remaining steps, after the actual immunohistochemical reaction, are to cover and seal the sample for preservation and long-term storage. The much more obvious method is to glue the cover to the sample using purpose-made resins that are commercially available.[(3)]

II. AIMS& OBJECTIVES

1. To compare the quality of antigen retrieval for Immunohistochemistry by clipping of slides and conventional method (Non clipping method).
2. To access the quality of Immunohistochemistry staining by clipping of slides method.
3. To access the quality of Immunohistochemistry staining by conventional pressure cooker method.

III. MATERIAL AND METHODS:

The study was carried out in the Department of Pathology, Adesh Institute of Medical Sciences and Research, Bathinda, Punjab, India.Total of 50 sections, 25 each of lymph node and skin biopsy sections with equal number of controls were subjected in this comparative study. A Comparative study design was used. The research was undertaken at the AIMSR, Department of Pathology, Bathinda, Punjab, India.

Inclusion Criteria:

1. From the histopathology section, all near normal lymph nodes and skin biopsy paraffin blocks, 25 each, were procured.
2. For study purposes, well preserved, well fixed and adequate tissue samples were used.

Exclusion Criteria:

1. Poorly preserved and poorly fixed lymph node tissue and skin biopsy sections.
2. Inadequate biopsy of tissues in the lymph node and skin.

Reagents

The primary antibodies were from the Biocare Medical company. Antibodies had already been diluted according to optimum concentration. Diva Decloaker, 20 X pH 7.0 diluted at a ratio of 1:20 ml (1ml of Diva buffer to 19 ml of distilled water) was the antigen retrieval solution used in the study. TBS Automation wash buffer, 40X, pH 7.7 diluted at 1:40 ml (1 ml TBS Automation wash buffer and 39 ml distilled water) ratio was being used shown in Plate1.Betazoid DAB Chromogen with chromogen DAB (3, 3 'Diaminobenzidine) had been used shown in Plate 6.

Technique:

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From lymph node and skin, 3-4 microns sections were collected. The tissue was deparaffinized and rehydrated in sodium citrate. The tissue containing the slides was overlapped with other plain slides as shown in Figure 1 by clipping one end using a steel paper clip(U clip of 2 Inches) before holding the slides in the buffer for heating in a temperature-controlled water bath for epitope unmasking/ retrieval for binding antibody as shown in Figure 2. Clipping was performed in only one end while the other end is slightly shifted laterally enabling the buffer to pass to the tissue and in order to make it easier to remove overlapping slides so that the tissue is not jammed between the two slides. Slides kept for boiling at 100 degrees Celsius for 15 minutes after that slides were removed from the water bath and allowed to cool at room temperature for 10 minutes and clips were removed. All at the same, tissue parts from the same lymph node and skin biopsy were kept in the pressure cooker for antigen retrieval and were used as a control shown in Plate 2 and 3 respectively.

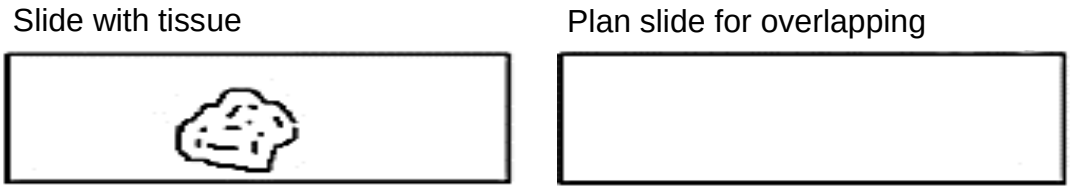

Figure 1: Slides before clipping

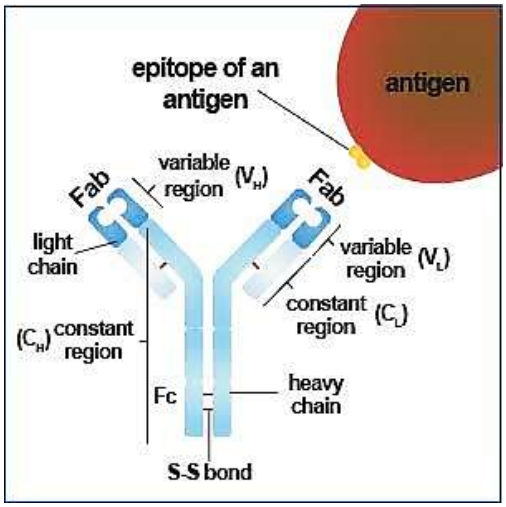

Figure 2: Epitope-antibody

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(a) (b)

Plate 2: Boiling beakers (a) Clipped slide in beaker (b). Heat induced antigen retrieval

(a) (b)

Figure 3: Pressure cooker (a) Non-clipped slide in pressure cooker (b) Heat induced antigen retrieval

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IHC staining:

Primary antibodies (Leucocyte common antigen for lymph node and for Squamous epithelium Pan Cytokeratin) were used. A comparison of tissue morphology and staining character, with or without clipping of slides was made. The primary antibody was incubated for a minimum of 1 hour and the secondary antibody was incubated at room temperature for 30 minutes to 1 hour. The slides were washed with TBS Automation wash buffer. By keeping for 15 minutes under DAB chromogen, the slides got stained with DAB chromogen. Before visualization the findings under a microscope, slides were then counterstained with hematoxylin and DPX mounting done.

Control slides:

There were no separate tissue sections of different organs been used. Experimental and controlled slides itself were used as auto controls with known antibodies. Leukocyte common antigen (LCA / CD45) for lymph node and Pan cytokeratin for skin biopsy sections.

Data handling:

The data was being recorded into the worksheet of Microsoft excel and examined using SPSS software.

Statistical method:

For statistical analysis, proportions and percentages were being used. The significance of the difference between the two attributes was evaluated using the method of Pearson chi-square. Significance level: P value < 0.05 was considered to be significant.

Independent Observers graded immunostaining on the basis of already laid criteria as:

1+ Faint staining in less than 40% of the cells - Poor

2+ Moderate staining in 40-80% of the cells - Satisfactory

3+ Strong staining in > 80% of the cells - Good

Similarly, antigen retrieval was also graded by independent observers on the basis of below mentioned criteria:

1+ Less than 50 % of tissue available after antigen retrieval method -Washed

2+ 50- 80% of original tissue intact - Folded/Damaged

3+ >80% of original tissue intact – Satisfactory

IV. RESULTS

Table 1: Distribution of sections on the basis of site of biopsy taken

Site	Number	Percentage
Skin	25	50.00%
Lymph node	25	50.00%

Above table shows the site from which the biopsy was taken and used in this study. A total of 50section of skin and lymph node were taken. Out of which 25 sections (50%) were of skin and 25 sections (50%) were of lymph node.

Table2: Comparison of antigen retrieval by gross observation of tissue by clipping vs non-clipping method

Antigen	Clipping	Non-Clipping
Satisfactory	**43**	**27**
Folded/Damaged	**4**	**11**
Washed	**3**	**12**

Chi square-6.48 df-1 p value-0.03

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Table 2 shows that by clipping method among 50 sections by gross observation, in 43 (86%) the tissue was satisfactorily retrieved, in 4 (8%) it was folded/damaged and in 3 (6%) cases it got washed.

In Non-clipping method 27(54%) were satisfactorily retrieved and 11 (22%) were folded/damaged and rest12(24%) were washed. These results are statistically significant.

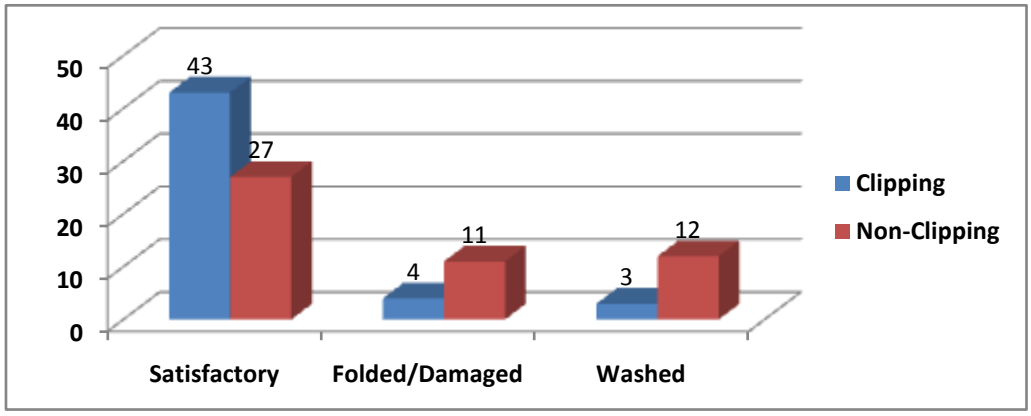

Figure 4: Graph showing antigen retrieval by gross observation of tissue byclipping vs non-clipping method

Table 3: Comparison of antigen retrieval by gross observation of clipping vs non-clipping method in skin biopsies

Antigen	Clipping	Non-clipping
Satisfactory	22	15
Folded/Damaged	1	7
Washed	2	3

Chi square-6.02 df-1 p value-0.04

Above table shows that by Clipping method among 25 of skin biopsy sections, in22 (88%) the tissue was satisfactorily retrieved, in 1 (4%) it was folded/damaged and in 2 (8%) cases it got washed.

In Non-clipping method 15 (60%) were satisfactorily retrieved, 7 (28%) were folded/damaged and rest 3 (12%) were washed. These results are statistically significant.

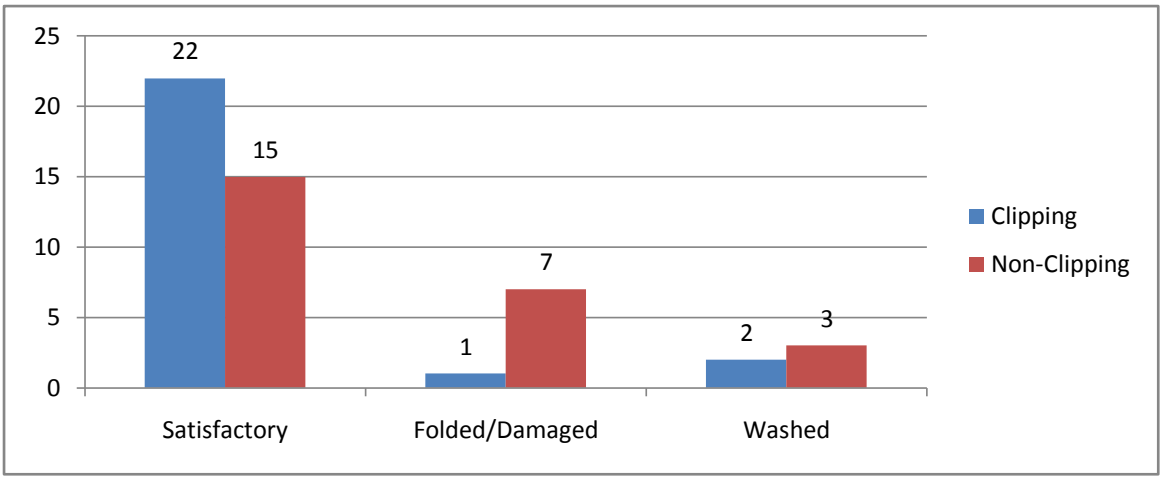

Figure 5: Graph showing comparison of antigen retrieval by gross observation of tissue by clipping vs non-clipping method in skin biopsies

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Table 4: Comparison of antigen retrieval by gross observation of tissue by clipping vs non-clipping method in lymph node biopsies

Antigen	Clipping	Non-Clipping
Satisfactory	**21**	**12**
Folded/damaged	**3**	**4**
Washed	**1**	**9**

Chi-square 8.9974. df-1 p-value is 0.01

Above table shows that by Clipping method among 25 of lymph node biopsy sections in 21 (84%) cases the tissue was satisfactorily retrieved, in 3 (12%) it was folded/damaged and in 1 (4%) cases it got washed.

In Non clipping method 12 (48%) were satisfactorily retrieved, 4 (16%) were folded/damaged and rest 9 (36%) were washed. The results are statistically significant.

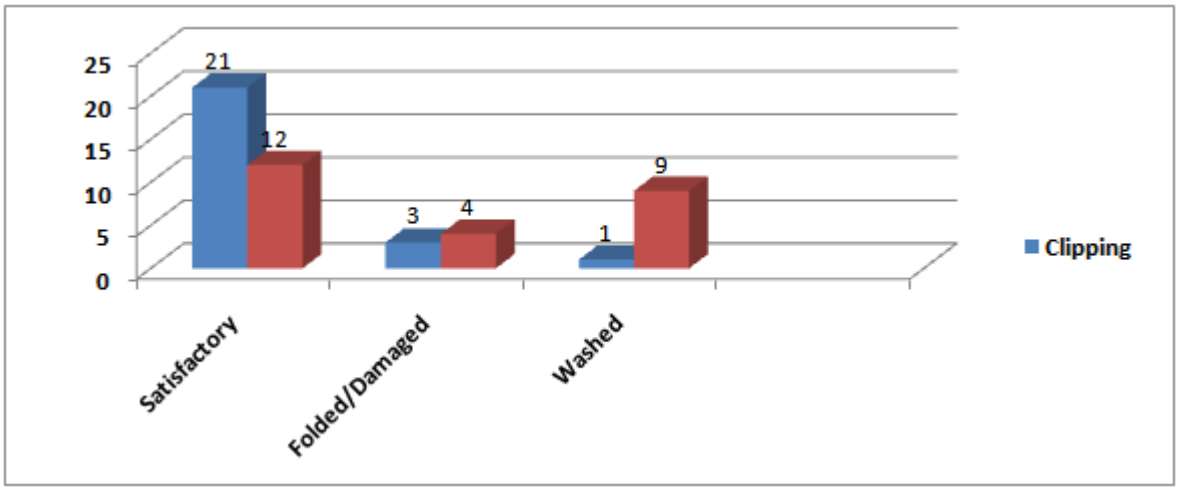

Figure 6: Graph showing comparison of antigen retrieval by gross observation of tissue by clipping vs non-clipping method in lymph node biopsies

Table5: Comparison of immunostaining by clipping vs non-clipping method

Staining	Clipping Method	Non-Clipping
Good 3+	**36**	**21**
Satisfactory 2+	**10**	**16**
Poor 1+	**4**	**13**

***** Based on the grading of immunostaining by independent observers.
Chi square-7.5 df-2 p value-0.02

Comparing the immunostaining by Clipping vs Non clipping method.

Among 50 cases majority of them i.e.36 (72%) had good immunostaining were by Clipping

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method, 10 (20%) were satisfactory and 4 (8%) had poor immunostaining.

Among Non-clipping method 21(42%) had good immunostaining,16 (32%) were satisfactory and 13 (26%) had poor quality of staining. These results are statistically significant.

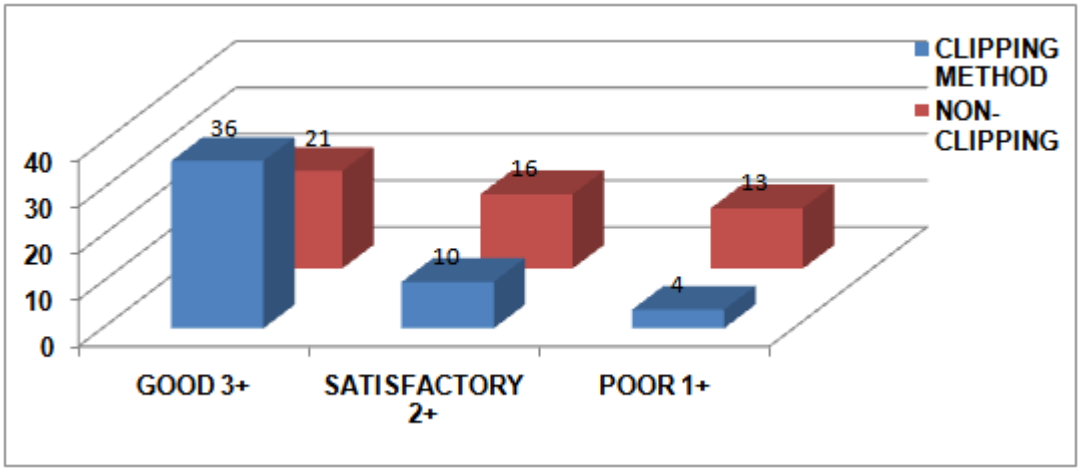

Figure7: Graph showing comparison of immunostaining by clipping vs non-clipping method

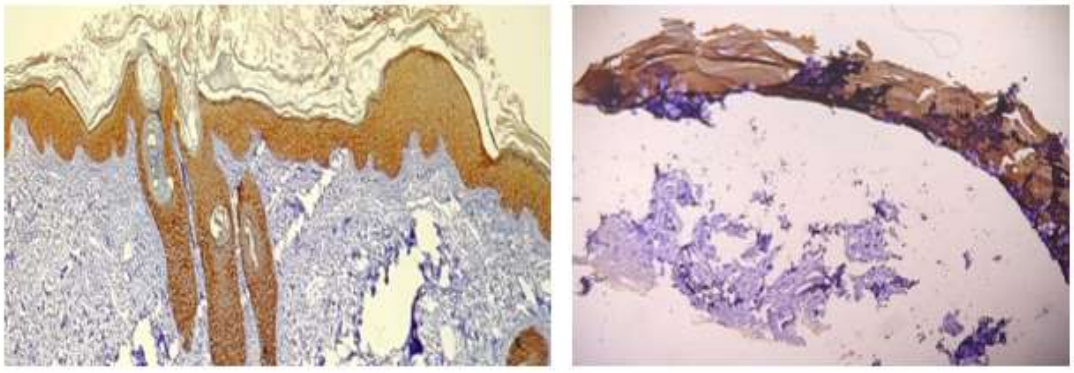

Figure 8: Skin Biopsy Section: (a) Clipping Method (b) Non-Clipping Method

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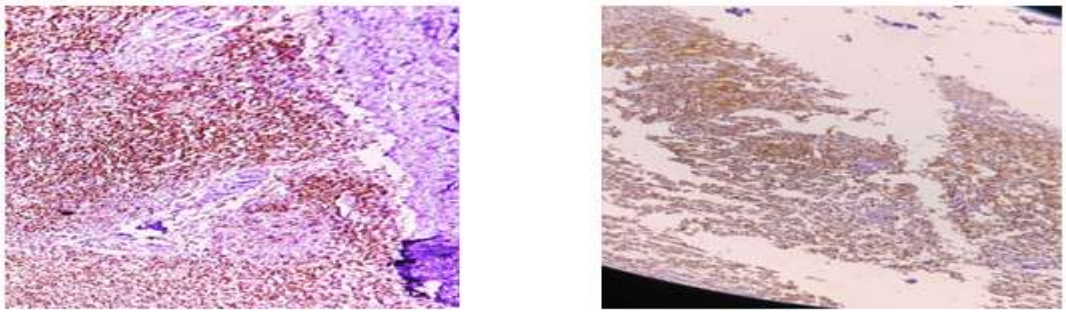

(a) (b)

Figure 9: Lymph Node Biopsy Section: (a) Clipping Method (b) Non- Clipping Method

Table6: Comparison of immunostaining by clipping vs non-clipping method in skin biopsies

Staining	Clipping Method	Non-Clipping
Good 3+	20	10
Satisfactory 2+	4	13
Poor 1+	1	2

* Based on the grading of immunostaining by independent observers.
Chi square-8.4314 df-2 p value-0.01

In 25 skin biopsy section by clipping method20 (80%) had good immunostaining, 4 (16%) were satisfactory and 1 (4%) had poor immunostaining.

Among Non-clipping method 10 (40%) had good immunostaining, 13 (52%) were satisfactory and 2 (8%) had poor quality of staining. These results are statistically significant.

Table7: Comparison of immunostaining by clipping vs non-clipping method in lymph node biopsies

Staining	Clipping Method	Non-Clipping
Good 3+	16	11
Satisfactory 2+	6	3
Poor 1+	3	11

* Based on the grading of immunostaining by independent observers.
chi-square 6.5928. df-1 p-value is 0.03

Comparing the immunostaining by Clipping vs Non clipping method in Lymph node

biopsies. Among 25 sections by clipping method 16 (64%) had good immunostaining, 6 (24%) were satisfactory and 3(12%) had poor immunostaining.

Among Non-clipping method 11 (44%) had good immunostaining, 3(12%) were satisfactory and 11 (44%) had poor quality of staining.The results are statistically significant.

V. DISCUSSION

Antigen retrieval is a significant step in immunohistochemistry. In expanding the scope and use of immunohistochemistry, the simple technique of boiling formalin-fixed paraffin embedded (FFPE) tissue sections in buffer has played a major role[4]. Direct boiling of tissues in the buffer, however also results in tissue damage or tissue falling off the slide. So many causes have been identified to contribute to the problem of dropping off or detaching tissues from slides, such as inadequate fixation, improper sectioning and drying, poor adherence and even uncleaned slides[5,6,7,8].

In a research done by Vinod KR in 2016 on a simple and efficient heat-induced antigen retrieval method, an additional step to the standard heat-induced antigen retrieval method was defined to improve the detection of antibody staining of formalin fixed paraffin embedded tissue. Direct tissue heating in the buffer is an effective method of epitope recovery, but it also results in tissue damage or failure. [9] In this process, Vinod.K.R overlapped the tissue section on the slide with a plain slide before keeping it in a heating buffer by clipping one end using a steel paper clip, maintaining a minimum gap between the slides. In this way, tissues heated in the buffer had the advantages over standard heat treatment for epitope recovery. Also at high temperatures, the tissues were intact, which increased the consistency of staining by avoiding folding, disruption or detachment of tissues from the slides.

Compared to approaches using a microwave (as shown in Plate 9) or pressure cooker our process is very safe and economical. In Microwave tissue section detachment from glass slide may occur during heating and can damage nuclear morphological details[10]. There is formation of hot and cold spots that leads to uneven antigen retrieval on the slide. [9]

In pressure cooker although it gives good results but direct boiling leads to folding or detachment of tissue. Clipped slides showed lesser damage/detachment or folding. Though pressure cooker has been found to be superior to microwave method in terms of antigen retrieval and safety but there has been cases of splash burn and projectile injuries[11]. These kinds of injuries are not seen in our technique.

PIER may lead to the alteration and destruction of the target molecule and tissue morphology also[12].Protease digestion can cause insufficient unmasking leading to false negative results while excessive digestion can adversely affect cytomorphological features and can cause detachment of tissues sections from slides[13].Compared to the other existing procedure, this simple additional technique tends to be very successful, safe and less time consuming. Clipping of slides has been effective step in antigen retrieval. Our study has given similar results as that of Vinod K.R.

In our study: A total of 50 sections were procured, 25 of which were taken each from skin and lymph node. Clipping of slide method was performed and subsequently CK and LCA(CD45) were used as primary antibodies for the skin and lymph nodes respectively.

By Clipping method among 50 sections by gross observation, in 43 (86%) the tissue was satisfactorily retrieved, in 4 (8%) it was folded/damaged and in 3 (6%) cases it got washed. In Non-clipping method 27(54%) were satisfactorily retrieved and 11 (22%) were folded/damaged and rest 12(24%) were washed.These results are statistically significant.

Among 25 of skin biopsy sections, in 22 (88%) the tissue was satisfactorily retrieved, in 1 (4%) it was folded/damaged and in 2 (8%) cases it got washed. In Non clipping method 15 (60%) were satisfactorily retrieved, 7 (28%) were folded/damaged and rest 3 (12%) were washed.The results are statistically significant.

Among 25 of lymph node biopsy sections in 21 (84%) cases the tissue was satisfactorily retrieved, in 3 (12%) it was folded/damaged and in 1 (4%) cases it got washed.Non clipping method 12(48%) were satisfactorily retrieved, 4 (16%) were folded/damaged and rest 9(36%) were washed.The results are statistically significant.

Among 50 cases majority of them i.e., 36 (72%) had good immunostaining were by Clipping method, 10 (20%) were satisfactory and 4 (8%) had poor immunostaining. Among Non-clipping method 21(42%) had good immunostaining,16 (32%) were satisfactory and 13 (26%) had poor quality of staining. These results are statistically significant.

By Clipping method in Skin biopsy sections, 20 (80%) had good immunostaining, 4 (16%) were satisfactory and 1 (4%) had poor immunostaining. Among Non-clipping method 10 (40%) had good immunostaining, 13 (52%) were

satisfactory and 2 (8%) had poor quality of staining. These results are statistically significant.

By Clipping method in Lymph node biopsy sections, among 25 sections 16 (64%) had good immunostaining by Clipping method, 6 (24%) were satisfactory and 3 (12%) had poor immunostaining. Among Non-clipping method 11 (44%) had good immunostaining, 3 (12%) were satisfactory and 11 (44%) had poor quality of staining. The results are statistically significant.

VI. SUMMARY:

Immunohistochemistry (IHC) incorporates histological, immunological and biochemical techniques by means of a particular antigen/antibody reaction marked with a visible label to distinguish specific tissue components. The IHC makes it possible to imagine the distribution and localization within a cell or tissue of particular cellular components. The method is typically used in tumor diagnosis as well as in non-neoplastic lesions. Semi-quantitative results of stained IHC markers are reported and have gained significant diagnostic and prognostic implications.

1. A number of 50 sections were procured, 25 of which were from skin and 25 from lymph node.
2. All the 50 sections were subjected to clipping method and simultaneously equal sections were run in pressure cooker as control.
3. By clipping method in 86% the tissue was satisfactorily retrieved, in 8% it was folded/damaged and in 6% cases it got washed. In Non-clipping method 54% were satisfactorily retrieved and 22% were folded/damaged and rest 24% were washed.
4. By clipping method in 25 cases of skin biopsy sections, in 88% the tissue was satisfactorily retrieved, in 4% it was folded/damaged and in 08% cases it got washed. In Non-clipping method 60% were satisfactorily retrieved, 28% were folded/damaged and rest 12% were washed.
5. By Clipping method among 25 cases of lymph node biopsy sections, in 84% the tissue was satisfactorily retrieved, in 12% it was folded/damaged and in 4% cases it got washed. In Non-clipping method 48% were satisfactorily retrieved, 16% were folded/damaged and rest 36% were washed.
6. Pan Cytokeratin and LCA(CD45) were the antibodies being used skin and lymph node antigen retrieval, specifically.
7. Among 50 cases majority of them 72% had good immunostaining were by Clipping method, 20% were satisfactory and 8% had poor immunostaining. Among Non-clipping method 42% had good immunostaining, 32% were satisfactory and 26% had poor quality of staining.
8. By clipping method among 25 cases of skin biopsy sections 80% had good immunostaining ,16% were satisfactory and 04% had poor immunostaining. Among Non-clipping method 40% had good immunostaining, 52% were satisfactory and 8% had poor quality of staining.
9. By clipping method among 25 cases of lymph node biopsy section 64% had good immunostaining by Clipping method, 24% were satisfactory and 12% had poor immunostaining. Among Non-clipping method 44% had good immunostaining, 12% were satisfactory and 44% had poor quality of staining.

VII. CONCLUSIONS

In this research, we have taken a further additional step towards the traditional method of heat-induced antigen recovery. In this changed process, we overlapped the tissue section on the slide with a plain slide before keeping it in a heating buffer by clipping one end using a steel paper clip, maintaining a minimum gap between the slides. Tissues heated in the buffer in this way had the following advantages for epitope retrieval over usual heat treatment. Also at high temperatures, the tissues were intact, which increased the consistency of staining by preventing tissue folding, damage or separation from the slides.

- Compared to the approaches using the microwave or pressure cooker, the process is very safe and economical.
- Compared to the currently existing methods, this simple approach also tends to be very successful and less time consuming.
- Hence moving on the conclusion that slides which are clipped have less chances of damage or destruction as compared to the non-clipped conventional method. Tissue sections exhibit good immunostaining by clipping method as compare to non-clipping method.

VIII. RECOMMENDATIONS

- We recommend this additional step of clipping and extending it in conventional pressure cooker method also.
- Clipping method can also be tried in cytology smears.

- Experimental studies where single panel testing has to be done i.e not on large scale, there our method can be used as a good option.
- Clipping method can be extended to frozen sections for the determination and comparison of IHC staining with HIER for further studies.
- At the time of concluding my thesis on Antigen retrieval by heat method a new instrument decloaking chamber had been introduced in our department of Pathology and the efficacy of clipping may be compared with this new instrument in future research.
- However more studies in this direction with larger sample size will direct the changes in the method of IHC.

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