

Immunohistochemistry in Developing Country – An Experience in Tertiary Care Hospital

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Abstract: *The immunohistochemistry technique is used in the search for cell or tissue antigens that range from amino acids and proteins to infectious agents and specific cellular populations. Immunohistochemistry is of paramount importance for the elucidation of differential diagnoses which are not determinable by conventional analysis with hematoxylin and eosin. It has been a recent addition to histopathologist and its establishment in a laboratory setting in a developing country where cost constraints play a major role along with skilled manpower, it becomes more challenging task. It is therefore important to share experiences of setting an Immunohistochemistry laboratory which will enable other upcoming laboratories to learn and disseminated knowledge.*

Keywords: immunohistochemistry, Breast Carcinoma.

1. Introduction

Histology is the microscopic study of cells and tissues by staining and examination under light/ electron microscopy. The purpose of staining is to highlight important features of tissues as well as to enhance the tissue contrast. Hematoxylin & Eosin is the most commonly stain used all over the world for histological examination.¹

There have been tremendous changes in histological techniques in past ten years, there are old stain procedures that are still in use today and many others have been replaced by new immunostaining.² Immunohistochemistry is a broad term that comprise of many methods used to determine tissue constituents (antigens) by using specific antibodies that can be visualized by staining.

Immunohistochemistry involves two steps³: a) Preparation of slide- This includes tissue processing, embedding, sectioning, antigen retrieval, non specific site block, endogenous peptide block, primary antibody incubation and detection. b) Interpretation of slides.

2. History of Immunohistochemistry

It began in nineteen forties when Marrack⁴ produced antigens against cholera & typhus using red stain conjugated to Benzidine tertaedro. There was rapid growth of Immunohistochemistry in nineteen sixties when Nakane⁵ introduced enzymes as marked antibodies. This took Immunohistochemistry to broader base as these could be interpreted using light microscopy and the requirement of fluorescence microscopy was not needed. There were series of development of unlabelled antibodies like Peroxidase-antiperoxidase by Sternberger⁶, Alkaline phosphatase anti alkaline phosphate (APAAP) by Masson et al⁷ that expanded application of Immunohistochemistry. It was during this period when diaminobenzidine molecule (DAB) got its use for the first time as conjugate in Immunohistochemistry which is used till date as a chromogen for peroxidase. The

next development was the discovery of antigen retrieval methods by Huang et al and secondary antibody detection methods by Hsu et al which made application of Immunohistochemistry in fresh specimens⁸. Immunohistochemistry plays a pivotal role in pathology and is referred to as 'Brown revolution' of histopathology⁹.

Uses of Immunohistochemistry in Pathology laboratory^{10, 11, 12, 13}

- Subtyping of lymphoma
- Characterization of primary site of malignancy
- Histogenetic identification of morphologically non differentiated neoplasm
- Differentiation of benign verses malignant tumors
- Prognostication of some disease

Sample Protocol¹⁴

A sample protocol which should give you an idea of the steps involved in performing an IHC.

Steps:

- 1) **Prepare:** Cut formalin fixed, paraffin embedded tissue between 3-5 microns thick. Mount on the desired slide of choice.
- 2) **Deparaffinzation** : It is done with either non-toxic solutions, or a series of alcohols and Xylenes.
 - a) Xylenes - 3 rinses - 1 minute each
 - b) Alcohols - 3 rinses - 1 minute each (100%, 70%, 30%)
 - c) Rinse with Distilled Water
- 3) **Epitope Retrieval:** Epitope retrieval applies heat with a pH solution to ensure that the epitopes are "retrieved", and the conformational changes incurred by paraffin are undone.
- 4) **Apply any Peroxidase** block or alkaline phosphatase block to your tissue, to prevent endogenous peroxidase or alkaline phosphatase from staining.
- 5) **Primary antibody**

- a) Dilute antibody in diluent (if provided in a concentrated format). Proceed to second step . if the antibody is a "predilute", or "ready to use".
- b) Apply your primary antibody (per specifications). Typically between 15 and 60 minutes.
- 6) **Detection:** Tag your antibody with an enzyme, which can either be horse-radish peroxidase or alkaline phosphates (HRP or AP).
- 7) Apply Detection (per specification). This is either biotin or polymer based, and can be one to two components (or in some instances more).
- 8) **Chromogen:** The chromogen binds to AP or HRP to initiate a color change, so that we can visualize it under a microscope.
- 9) Apply your chromogen (Typically 5 - 20 minutes)
- 10) **Counterstain:** The counter stain allows us to see the rest of the cells in the tissue samples that do not react with the chromogen. The most common is Hematoxylin, however other counterstains can be used as well. Apply Counterstain (1 - 10 minutes)
- 11) **Mount & Cover slip**

3. Our Experience

Immunohistochemistry is a relatively simple technique but its ability to resolve issues depend on experience of hands that perform it and eyes that interpret the results. Although a wide variety of protocols for standardizing the Immunohistochemistry technique are being proposed individual laboratories must establish a standardized procedures, validate their findings.

In a developing country where we need to maintain a balance between patient need and affordability it becomes important for laboratories establishing Immunohistochemistry protocols to take baby steps without compromising the quality and spending economically. We started Immunohistochemistry in 2012 and it has been five years now. Initially starting with ER,PR and HeR 2 Neu staining gradually expanding the number of markers . It was a tremendous learning experience over these five years. We would like to share our experience with our fellow colleagues in the field of Immunohistochemistry and especially those who plan to start up Immunohistochemistry laboratory in their setting.

The specimen fixation was done in 10% buffered Formaldehyde solution. Tissue processing was done at temperatures less than the melting point of wax in a range of 52 -60 degree Celsius. It also depends on the manufacturer specification. Nevertheless, temperature of processing was kept under 60 degrees Celsius so that the specimen antigenicity was not compromised. The thicknesses of sections were kept at 4 um as further thinning of sections could have resulted in weak immunostaining.

We avoided taking frozen tissues for routine Immunohistochemistry. Antigen retrieval was done by microwave technology with controlled humidity and pressure. We used Avidin- biotin-peroxidase complex (ABC) as detection system (secondary antibody). Antibody panel was selected judiciously and a specialized technician dedicated to Immunohistochemistry was trained. The

incidence of crossed immunological reaction was kept at minimal. The choice of primary antibody selection (Monoclonal or Polyclonal) was dictated as per availability in market. The background false positivity was taken care of by using sniper.

Initially we started with ER/PR & Her 2 Neu detection on breast carcinoma cases. It took us nearly ten months for standardization and validation of our findings. Once established we started taking cases in 2011 and in further years we incorporated variety of marker like Cytokeratin, Desmin, Vimentin, synaptophysin, HMB-45, CCA, MIC-2, round cell tumor markers and lymphoma panel. As the clinicians gained confidence in our reports the number of case rose from 473 in 2011 to 652 in 2014, 1100 in 2015 and 1500 in 2016. (Figure 1)

It is of utmost importance to know the diagnostic sensitivity and specificity of each marker employed. Before deciding on the choice of panel. In initial phases always begin with limited panel of markers and gradually add on second line markers. Regular update of Immunohistochemistry markers with new data is essential as it is a dynamic ever growing field.

The interpretation of Immunohistochemistry expression is done by qualitative presence or absence of brown staining. All brown staining are however not positive as one must be aware of location of target molecule- nuclear or and cytoplasmic while reporting the result.

Year	Case	Antibody panel
2012	348	ER, PR, Her2 Neu
2013	473	ER, PR, Her2 Neu , Cytokeratin, HMB 45, LCA, MIC-2, Desmin, Vimentin
2014	652	ER, PR, Her2 Neu , Cytokeratin, HMB 45, LCA, MIC-2, Desmin, Vimentin Round cell tumor markers
2015	1100	ER, PR, Her2 Neu , Cytokeratin, HMB 45, LCA, MIC-2, Desmin, Vimentin Round cell tumor markers Lymphoma panel
2016	1500	ER, PR, Her2 Neu , Cytokeratin, HMB 45, LCA, MIC-2, Desmin, Vimentin Round cell tumor markers Lymphoma panel

Figure 1

Marker	Frequency	Percentage
ER+/PR+	60	36.4
ER+/PR-	5	3.0
ER-/PR+	9	5.5
Her 2 Neu+	67	40.6
Triple Negative	5	3.0
Triple Positive	19	11.5
	165	100

Figure 2

We conducted a study of all modified radical mastectomy specimens together with core breast biopsies specimen diagnosed as malignant over a period of three months in the department of Pathology, BMCHRC, Jaipur. The paraffin embedded tissue sections were stained with Hematoxylin & Eosin and reported on routine histopathology. IHC was performed by peroxidase- antiperoxidase technique following the above mentioned protocol and reporting was

done by two independent Pathologists. ER, PR positivity was denoted by nuclear staining using Allred scoring system taking into account both intensity of staining and percentage of positive tumor cells. For reporting Her 2 Neu American society of clinical oncology guidelines 2007 were taken as reference. The descriptive statistical analysis is given in table -2.

Her 2 Neu positivity was seen in 40.6 % (67) patients, ER & PR positivity was seen in 36.4 % (60). 11.5 % (19) cases were triple positive where as 3.0 % (5) cases were triple negative. In 3 % (5) cases ER positivity was noted with PR negative. 5.5 % (9) cases were PR positive and ER negative.

IHC is of paramount importance in treatment planning of patients with breast carcinoma. Each laboratory should standardize their procedures for IHC to establish this core stone facility.

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