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HAEMATOXYLIN AND EOSIN STAINING IN THE DIAGNOSIS OF HODGKIN'S DISEASE IN UGANDA

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ABSTRACT

Background: **Whereas immunohistochemical methods have been widely used for the diagnosis and classification of Hodgkin's disease in the developed countries, there are very few reports of their use in the developing countries where haematoxylin and eosin is the mainstay of diagnosis of Hodgkin's disease. Yet the diagnostic accuracy of haematoxylin and eosin has not been assessed in Uganda.**
Objective: **To determine the reliability of haematoxylin and eosin staining in the diagnosis of Hodgkin's disease using immunohistochemistry as the reference standard.**
Design: **Laboratory based cross sectional study.**
Setting: **Makerere University Medical School, Department of Pathology.**
Methodology: **Two hundred and forty formalin fixed, paraffin embedded biopsies seen in the Makerere University, Department of Pathology from 1980-2000 were studied. The tissue sections, were assessed and subjected to immunohistochemical methods using monoclonal antibodies including leucocyte common antigen, LCA (CD45), antibodies to Reed-Sternberg cells (CD15, CD30) and antibodies to B cells (CD20). The sensitivity, specificity, positive predictive value and negative predictive value were assessed. The overall Kappa score was used to assess the agreement between the two diagnostic tests.**
Results: **Of the 240 biopsies, 171(71.3%) were confirmed as Hodgkin's disease by immunohistochemistry. Using haematoxylin and eosin (H&E), only 131 of the 171 cases of Hodgkin's disease were detected. The mean age of the 171 cases was 26.1 (SD 16.2) years, with a mode of 20.0 and median of 22.5 years. The 15-24 year age group was the most affected (47.2%). There were more males (65.9%) than females and most were Baganda the dominant tribe in the central region. The sensitivity, specificity, positive and negative predictive values of haematoxylin and eosin were 76.61%, 92.75%, 96.32% and 61.53% respectively. The agreement between the two tests was 81.25% with an overall measure of agreement, Kappa, of 0.602.**
Conclusion: **Haematoxylin and eosin has relatively high efficacy in the diagnosis of Hodgkin's disease. Use of haematoxylin and eosin is still recommended for the diagnosis of Hodgkin's disease, reserving the expensive immunohistochemistry for difficult cases.**

INTRODUCTION

Since the discovery of Hodgkin's disease by Dr. Thomas Hodgkin in 1832, there has been an explosion of knowledge regarding aetiology, diagnosis and treatment throughout the world(1). Despite this, diagnosis of Hodgkin's disease in the developing countries is still largely based on haematoxylin and eosin staining. Hodgkin's disease is defined histologically by complex and variable morphology in which the malignant Reed-Sternberg (RS) cells are found amidst lymphocytes, plasma cells, eosinophils and histiocytes(2-3).

Hodgkin's disease ranks third among malignant lymphomas in Uganda (after Burkitt's lymphoma, and other non-Hodgkin's lymphomas), according to the Kampala Cancer Registry(4). Its incidence seems to have remained static in contrast to other lymphomas in which there has been a dramatic rise in incidence since the beginning of the HIV/AIDS pandemic(5).

The prognosis of Hodgkin's disease largely depends on accurate histopathological diagnosis and classification(6). However histopathological diagnosis poses some problems for the histopathologist when the classical histological features of Hodgkin's disease are not seen(6).

Even in expert hands, histopathological diagnosis for Hodgkin's disease can be erroneous as demonstrated by Wright(7) in a study carried out in the Makerere University Department of Pathology more than thirty years ago. In this study Wright found that 20% of the cases diagnosed as Hodgkin's disease had, in fact, other diagnoses such as reactive lymphadenopathy, anaplastic

metastatic tumour and histiocytic lymphoma. In a similar study by Miller(8) and colleagues, 13% of 287 cases initially diagnosed as Hodgkin's disease had been misdiagnosed. In the developed countries immunohistochemistry is being used for diagnosis in problematic, borderline and in unusual cases to resolve the diagnosis. However, in resource poor countries such as Uganda, haematoxylin and eosin staining remains the mainstay of diagnosis.

Haematoxylin and eosin staining: This is the most popular routine stain in the histological laboratory. It has the advantage of selectively staining the cell nuclei, cytoplasm and connective tissue with the nuclei appearing blue and the latter varying shades of pink(9).

The principle of staining: Haematoxylin is an acidic dye that stains the acid content of the nucleus in the presence of a mordant such as aluminium, potassium alum, tungsten or iron. The mordant forms a link called "a lake" between the nucleus and the dye and this attachment is permanent(10). Eosin is a red dye, which stains the cytoplasm and connective tissue in varying shades of pink giving a most useful differential stain(11).

The role of immunohistochemistry: Immunohistochemical techniques are used to detect antigenic molecules *in situ* in human cell and tissue samples, and have been used widely in recent years both in diagnostic pathology and in research into human disease.

Monoclonal antibodies (antibodies of single specificity, generated from immortalization of plasma B cells *in vitro*) are essential tools for detecting antigenic molecules.

The antigenic molecules on lymphocytes were defined in a series of international workshops and the 1987 workshop assigned the CD or 'cluster of differentiation' designation(11). These are in essence clusters of antigens. This CD designation was based on the use of sets of monoclonal antibodies, from different laboratories, that reacted with specific surface antigens. Monoclonal antibodies (antibodies of single specificity, generated from immortalization of plasma B cells *in vitro*) are essential tools for detecting antigenic molecules. The data were then collected from different laboratories and the cell surface antigens were delineated to specific CD numbers(11). Todate over one hundred CD antigens have been defined.

Immunohistochemical studies have mapped the expression of various CD antigens to specific cells and tissues. This has led to their use as cell or tissue markers in the histopathology diagnosis of lymphomas. Immunophenotype refers to the antigenic profile for example B cells express CD20 and CD79a. Many studies have been done in western countries on immunophenotype of Reed-Sternberg (RS) cells in an attempt to define the origin of these cells (12-14). In fact some recent studies have defined the origin of RS cells as B-lymphocytes (15,16).

For the diagnosis of Hodgkin's disease, combinations of a number of markers are used. These include activation antigens (CD30, CD15, CD25 and CD71), HLA related markers (CD74, HLA-DR), T and B cell associated antigens UHCL-1/CD7 and CD20/L26 and leucocyte common antigen (CD45).

More recently Carbone and others (17) have shown that RS cells and their morphologic variants express CD40 in all the 171 cases of Hodgkin's disease studied irrespective of the histological types.

Monoclonal antibodies have also been used to distinguish Hodgkin's disease from other lymphomas that histologically show a striking similarity to Hodgkin's disease such as T-cell/histiocyte-rich B cell lymphoma and anaplastic large cell lymphoma. Hodgkin's disease is an important neoplasm whose diagnosis has moved from only routine histologic techniques (commonly used alone in the developing countries) to the use of immunohistochemistry and molecular methods in the developed countries.

While conventional histologic techniques are the mainstay of diagnosis and classification of Hodgkin's disease, the degree of disparity using these methods is not known as reported by Wright(7), Miller *et al*(8) and Symmer(18).

Whereas immunohistochemical methods have been widely used for the diagnosis and classification of lymphomas in the developed countries (19, 20), there are very few reports of their use in the developing countries(21). In the developed countries these methods have been found to be more accurate than routine microscopy (22,23). In the developing countries such as Uganda, pathologists still rely on routine histologic techniques, yet the diagnostic accuracy of these methods has not been assessed in our environment.

General objective: The general objective of this study was to determine the reliability of haematoxylin and eosin staining in the diagnosis of Hodgkin's disease in Uganda.

Specific objectives: To determine the specificity and sensitivity of haematoxylin and eosin staining in the diagnosis of Hodgkin's disease and to determine the positive and negative predictive values in the diagnosis of Hodgkin's Disease.

MATERIALS AND METHODS

Study design: This was a laboratory-based cross sectional study to evaluate immunohistochemistry against haematoxylin and eosin staining as a diagnostic method during a fixed period (1980-2000).

Setting: The study was carried out at the Makerere

University Medical School, Department of Pathology, which renders the bulk of biopsy services for the whole country and autopsy services for Mulago Hospital handling over 6000 biopsies per year. All the biopsies received from hospitals and health units including Mulago Hospital are kept as formalin-fixed, paraffin embedded tissue blocks.

Study population: This consisted of biopsy specimens received and preserved as formalin-fixed, paraffin embedded tissues, in the Makerere University Department of Pathology, from 1980-2000 inclusive.

Sampling procedure: The first 200-biopsy specimens where a diagnosis of Hodgkin's disease was made from 1980 to 2000 inclusive and the first 40 biopsy specimens of reactive lymphadenopathy during the same period were consecutively entered into the study.

Sample size estimation: Several approaches are recommended in estimating sample size in reliability studies. Standard chart, can be used for minimum sample size based on calculations of reliability coefficients. It is recommended that the study involve at least 200 subjects (24-25). For the current study a minimum sample size of 200 subjects was used.

Selection Criteria

Inclusion criteria: All biopsies where a diagnosis of Hodgkin's disease was made by a histopathologist from 1980-2000 inclusive.

Exclusion criteria: Biopsies with poorly fixed sections, sections with extensive tissue or cell necrosis, specimens sent in the form of aspirates.

Data collection methods: The specimens for the study were retrieved from the general pool of formalin- fixed, paraffin embedded tissue blocks in the Department of Pathology. They were screened and assessed; and those meeting the inclusion criteria were entered into the study. Each specimen was given a serial number and tissue blocks were then subjected to routine H&E and re-interpreted by the investigator using the criteria proposed by Lukes and Butler and the Rye conference (26-29). Where it was not clear as to whether the diagnosis was nodular sclerosis Hodgkin's disease, Gordon and Sweets' reticulin method(10) for reticulin fibres was employed to verify this. The histologic diagnosis of each specimen was entered on to a data entry form. The same tissue blocks were subjected to monoclonal antibodies CD 15, CD20, CD30 and CD45 and an immunohistochemical diagnosis made(27). The results for each specimen were entered on to the data entry form.

Data management and analysis: Data were collected on data collection forms and entered into the computer using EPI INFO software for storage and initial analysis. Further analysis was done using SPSS and Medcal. Exe software. The data were summarised in frequency tables, means, graphs and charts. The sensitivity, specificity, negative and positive predictive values of haematoxylin and eosin staining were calculated using two by two tables. The level of agreement between the two diagnostic tests was assessed by the overall Kappa score.

Quality Control: For effective quality control, positive and negative controls for the immunostains were used. The positive controls were provided as unstained slides by DAKO Denmark for each of the monoclonal antibodies, that is CD15, CD20, CD30 and CD45. The negative control was a slide of reactive lymphadenopathy. The investigator and one laboratory technician prepared and stained the slides.

The stains were used in the shortest time possible to avoid deterioration. The microscope used was checked daily and cleaned. One of us (LK) was blinded to the original detailed histological diagnosis, by mixing 40 blocks of reactive lymph nodes with the 200 blocks of Hodgkin's disease. These were processed together and subjected both to H&E and immunostains. The opinion of the two senior pathologists was sought before the final diagnosis was decided on for both the immunological and histopathological stains.

The slides were interpreted by LKT with the help of the senior pathologists. The diagnosis of Hodgkin's disease was first made using light microscopy using the criteria proposed by Lukes and Butler(28) and the Rye Conference(29).

Immunohistochemical diagnosis: The diagnosis of Hodgkin's disease was confirmed by staining the sections with monoclonal antibodies(27), which bind to antigens expressed on the Reed-Sternberg cells. Panels of four antibodies were used including antibodies to CD15, CD30, CD45 and to B cell antigen CD20.

Ethical consideration: Permission to carry out the study was obtained from the Department of Pathology, the Makerere Faculty of Medicine Research Committee and the National Council of Science and Technology. For confidentiality, the names of the patients were not used; instead the specimens were identified by the biopsy and unique identification numbers. No informed consent was sought and there were no potential risks to the patients from whom the biopsies were taken. Where relevant, the investigator informed the physician in the event of a change in the diagnosis.

RESULTS

Socio demographic characteristics of study population: The age distribution of the 171 patients, confirmed as having Hodgkin's disease by immunohistochemistry is shown in figure 1. The majority of patients with Hodgkin's disease were in the 15-24 year age group with a mean of 26.1 and standard deviation 16.2 years. There were 112(65.9%) males and 58(34.1%) females with Hodgkin's disease during the period of review, showing a male to female ratio of 1.93:1. One patient had no sex recorded.

Specificity and sensitivity of haematoxylin and eosin using the immunohistochemistry as gold standard: The sensitivity, specificity, NPV and PPV were computed using information in Table 1.

Table 1

Test results of haematoxylin and eosin against immunohistochemistry as the gold standard

		Immunohistochemistry		
		Positive	Negative	Total
Haematoxylin & eosin	Positive	131	5	136
	Negative	40	64	104
Total		171	69	240

The sensitivity of haematoxylin and eosin staining was 76.61%; the specificity 92.75%, positive predictive value 96.32% and negative predictive value was 61.53%. The agreement between haematoxylin and eosin staining and immunohistochemistry was 81.25% with an overall Kappa score of 0.602.

Of the 40 specimens testing positive on immunohistochemistry, 31(77.5%) were diagnosed as reactive lymph node by haematoxylin and eosin stain. Others included nasopharyngeal carcinoma (7.5%), secondary carcinoma to the lymph node (7.5%) and others (7.5%).

DISCUSSION

The aim of this study was to determine the reliability of haematoxylin and eosin staining in the diagnosis of Hodgkin's disease in Uganda. Specifically the study was designed to determine the sensitivity, specificity, positive and negative predictive values of haematoxylin and eosin staining in the diagnosis of Hodgkin's disease.

Sensitivity and specificity: In this study 240 formalin-fixed, paraffin-embedded tissue blocks from the period 1980-2000 were retrieved from the repository and new sections were made, processed and subjected to both haematoxylin and eosin and immunohistochemistry stains.

One hundred and seventy one (71.25%) cases had Hodgkin's disease, while 69(28.75%) were negative. Haematoxylin and eosin staining did not detect forty or 23.4% of the 171 cases with Hodgkin's disease.

The sensitivity of haematoxylin and eosin staining was 76.6% while the specificity was 92.75%. The sensitivity of haematoxylin and eosin is relatively low making the method not such a good screening test for Hodgkin's disease. In a similar study, Jones and colleagues (30) found a sensitivity of 93.0%, which is higher than the 76.6% recorded in the current study. The possible reason for this difference in sensitivities could be due to the fact that the diagnoses in Jones and colleagues report were confirmed after review by a national panel of haematopathologists in an elaborate process of consultation(30).

The specificity of haematoxylin and eosin staining in the current study was high (92.8%). This means that haematoxylin and eosin staining is good at detecting tissues without Hodgkin's disease. This contrasts sharply with the very low specificity (8%) found in the study by Jones and colleagues(30). In a related study, Wright(7) established that, even in expert hands, histopathological diagnosis of Hodgkin's disease was found erroneous in 20% of cases initially diagnosed as Hodgkin's disease. The specimens studied by Wright(7) had been assigned other diagnoses such as reactive lymphadenopathy, anaplastic metastatic tumour and histiocytic lymphoma,

Indeed in the current study 4% of the cases diagnosed as Hodgkin's disease did not have it. Instead they had diverse diagnoses ranging from reactive lymphadenopathy to anaplastic large cell lymphoma which is CD30 positive.

This finding is comparable to that of Miller and colleagues(8), who found that 13% of 287 cases had been initially mis-diagnosed as Hodgkin's disease. In that study, an expert panel of haematopathologists established that mistakes had been made because of the confusion between other malignant lymphomas with Hodgkin's disease especially large cell lymphomas with pleomorphic features and Reed Sternberg-like cells (8). In fact mixed cellularity and lymphocyte depleted Hodgkin's disease were the most frequently mistaken cell types while those with nodular sclerosing type were the least likely to be confused with other neoplasms.

The findings of this study compare favourably with the results of a study by Symmers in which up to 47% of 600 specimens initially diagnosed as Hodgkin's disease had mistaken diagnosis ranging from chronic lymphadenitis to reticulum cell sarcoma(18).

Positive and negative predictive values: The positive predictive value was 96.3% while the negative predictive value was 61.5%. The positive predictive value of 96.3% is the probability of the pathologist, using haematoxylin and eosin staining, of making a correct diagnosis of Hodgkin's disease. The negative predictive value on the other hand means that the probability of the pathologist excluding Hodgkin's disease was 61.5%. These figures are comparable to those in the study by Glaser *et al*(3). These authors were able to calculate different predictive values for each histologic subtype since the numbers studied enabled them to do that. For example their positive predictive value for nodular sclerosis subtype was 95% and for lymphocyte predominance was 69%.

Level of agreement between the two tests: The level of agreement between the two diagnostic tests, haematoxylin and eosin staining and immunohistochemistry using a panel of antibodies was quite high at 81.3%. The overall Kappa score was found to be 0.602 and indicated that the reproducibility of the current results is good since Kappa lay between 0.4 and 0.75. The results compare well with the Kappa value of 0.66 obtained in the study by Glaser *et al*(3).

In the current study, five of the 69 cases classified

as not having Hodgkin's disease by immunohistochemistry were wrongly diagnosed as having it by haematoxylin and eosin staining. These cases in fact had different diagnoses; two were reactive lymph nodes and three were anaplastic large cell lymphomas, which tested $CD30^+$.

It is not unusual to confuse large cell lymphomas with Hodgkin's disease since these lymphomas have Reed-Sternberg-like cells(2). Immunophenotyping is critical for distinguishing Hodgkin's disease from anaplastic large cell lymphoma; both express CD30, but anaplastic large cell lymphoma rarely expresses CD15. In the current study it was not possible to do further tests (CD40, CD26) to distinguish between these two entities, as has been done by Carbone and others(19), because the suppliers did not have them. Efforts to get the antibodies from Carbone and others in Italy were futile.

Forty(23.4%) of the 171 cases of Hodgkin's disease were not detected by haematoxylin and eosin. Thirty five came from the pool of 200 cases of Hodgkin's disease sampled and five from the pool of 40 cases of reactive lymphadenopathy. Instead they had been assigned other diagnoses such as reactive lymph node (77.5%), nasopharyngeal carcinoma (7.5%) and others.

Conclusions and recommendations: With a sensitivity of 76.6%, specificity of 92.8% and Kappa of 0.602, haematoxylin and eosin has relatively high efficacy in the diagnosis of Hodgkin's disease. Use of haematoxylin and eosin is still recommended for the diagnosis of Hodgkin's disease reserving the expensive (USD 20 per test) immunohistochemistry for difficult cases.

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