Recent Methods and Techniques in Diagnostic Histopathology: The Impact on Tropical Pathology Practice

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Abstract

***Background*:** *The practice of diagnostic histopathology in the Tropics is beset by numerous challenges, including a dearth of anatomical pathologists, and inability to retain both locally and foreign-trained pathologists. Several inhabitants of the region have no access to any form of healthcare, let alone pathology services. This review article examines trends in the practice of histopathology in a developing country in the Tropics.*

Materials and methods: *A critical review of the MEDLINE and other online and published resources was undertaken.*

Results: *The role of various diagnostic tools in autopsy and surgical pathology are outlined and the current availability of these modalities is discussed. The haematoxylin-eosin method remains the basic minimum for histological diagnosis, assisting in the choice of more specific techniques. Histochemical staining methods have a critical role to play in several diagnostic scenarios, particularly in the resource-limited setting of third world economies. There is a trend of increasing availability of hands-on immunohistochemical staining in several indigenous histopathology laboratories. However, more sophisticated capital intensive methods such as electron microscopy, flow cytometry, and molecular genetics procedures are still largely research methodologies, even in the most developed countries, and remain outside the scope of routine diagnostic practice in the tropical setting for a long while yet.*

Conclusions: *It is hoped that newly discovered surrogate immunohistochemical markers for specific genes will in the near future be added to the arsenal of the routine histopathology laboratory of developing countries such as Nigeria.*

Keywords: *Histopathology, Tropics, Autopsy, Surgical Pathology*

Introduction

Pathology is the clinical diagnostic science that underpins patient care[1]. For the purpose of this review article, we shall primarily be focussing on trends in the practice of anatomical pathology (histopathology), with respect to what currently obtains in developing countries in the tropics, using Nigeria as our reference point.

There are an estimated nine million physicians worldwide, out of which the vast majority reside in developed countries[2]. There are currently only 39,000 doctors in the West African sub region as compared to the 280,000 actually required. In Nigeria, the physician to general population ratio is only 0.27 per 1,000, which, if anything, has declined from the ratio of 1 in 30,000 (0.33 per 1,000) cited by Edington *et al* almost 40 years ago[3].

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It has been predicted that by 2015 there shall be a critical shortage of doctors of all cadres throughout sub-Saharan Africa[4]. Major issues confronting laboratory physicians in sub-Saharan Africa include the shortage of pathologists, especially anatomical pathologists, inability to retain pathologists in the public service and the exodus of both locally and foreign-trained pathologists to "greener pastures"[5].

Another peculiarity of healthcare delivery in sub-Saharan Africa is the lack of access of the general population to any form of healthcare, let alone pathology services[5]. The laboratories and health services of the developing world account for a mere 5% of the world's consumption of laboratory instrumentation and reagents[6].

Diagnostic histopathology practice

Histopathology as a clinical practice broadly encompasses autopsy and surgical pathology. The primary roles of the autopsy in medical practice have been reviewed in detail elsewhere[7]. In spite of the numerous benefits of autopsy examination, there has been a decline in post-mortem rates in developing countries. A recent cross-sectional survey from Ibadan, Nigeria revealed that only 38% of relatives and 50% of doctors had satisfactory knowledge and attitudes regarding autopsy practice[7]. Difficulty in obtaining consent from relatives, administrative problems, and delayed autopsy reports were identified as major impediments. Relatives of the deceased refused consent for autopsy on the grounds of fear of mutilation, delaying the funeral, and objection by the patient before death[7]. Delay in performing autopsies is contributed to by the frequent difficulty of clinicians' seeking consent from the next of kin immediately after bereavement and is often exacerbated by additional factors such as lack of electricity or water.

The unhealthy trend of declining autopsy rates needs to be urgently addressed by all stakeholders in the medical profession, with pathologists serving as the vanguard. The lay public, other medical doctors, ancillary medical staff, hospital administrators and the state and federal government need to be constantly informed about the role of the autopsy[7].

Surgical pathology provides definite histological diagnosis, facilitating choice of appropriate therapy, formulation of prognosis of ongoing disease processes, and monitoring of the outcome of therapy.

Tools of diagnostic histopathology

Diagnostic histopathology in the tropics is largely restricted by the widespread unavailability of all but the most basic tools due to lack of investment in the infrastructure and reagents that are required, and in many cases lack of dedicated trained ancillary staff.

Diagnostic histopathology uses the following tools and methods for morphological diagnosis on surgical and post-mortem tissue biopsies:

1. Haematoxylin-eosin
2. Histochemical stains
3. Immunohistochemical stains
4. Electron microscopy
5. Flow cytometry
6. Molecular techniques

1**.** *Haematoxylin-eosin*

This technique is the basic minimum for histological diagnosis. With careful clinicopathological correlation, the majority of histological diagnoses, or in most other cases, a list of differential diagnoses that can guide clinical and laboratory investigations and treatment can be arrived at confidently. In general, haematoxylin-eosin serves as an invaluable barometer for determining the line of clinical management. For example, in the case of neoplasms, histological typing, histological grading and other histological parameters of prognostic significance (e.g. tumour margins, mitotic counts, pathological tumour staging and lymphovascular invasion) are primarily ascertained using haematoxylin-eosin stained preparations. The advantages of

haematoxylin-eosin include its cost effectiveness, universal availability and the simplicity of the technique. With experienced and competent laboratory staff, this indispensable technique can assist greatly in the resolution of most important clinicopathological issues.

staining methods are not routinely performed. Special stains have a critical role to play in several diagnostic scenarios, particularly in the resource-limited setting of third world economies. These techniques may be applied both to histological sections and to cytology smears, with good results[8].

Table 1.

List of selected special stains routinely employed in Diagnostic Histopathology

Histochemical target	*Special stains*
1. Organisms	
Mycobacteria	Ziehl-Neelsen
Bacteria	Tissue Gram e.g. Brown-Hopps
Fungal organisms	Periodic acid Schiff (PAS), Grocott
Helicobacter pylori	Giemsa, Dieterle, Warthin-Starry
Hepatitis B (surface antigen)	Shikata orcein
2. Connective tissue matrix	
Collagen	Masson trichrome, Van Gieson
Elastic fibres	Verhoeff Van Gieson, orcein
Mucopolysaccharides	Alcian blue
Basement membrane	Jones' methenamine silver
3. Pigments and deposits	
Melanin	Fontana-Masson
Haemosiderin	Perls' Prussian blue
Calcium	Von Kossa, Alizarin red
Amyloid	Congo red
4. Cytoplasmic constituents	
Glycogen	PAS with diastase
Lipid	Sudan black
Argyrophil granules	Grimelius
Argentaffin granules	Fontana-Masson
Muscle filaments	Phosphotungstic acid haematoxylin
Skeletal muscle	Gomori (reverse) trichrome
Myelin	Luxol fast blue
5. Enzyme histochemistry	
Skeletal muscle	ATPase, NADH dehydrogenase
Melanocytes	DOPA reaction

2. *Histochemical stains*

Histochemical (special) stains rely on chemical interaction between dyes and tissue matrix or cell components or products. It is disheartening that in many secondary and some tertiary centres in tropical Africa, simple histochemical

Among some of the applications of special staining are demonstration of organisms, connective tissue matrix components, pigments and other extracellular deposits, cytoplasmic constituents and enzyme histochemistry (Table 1). The major limitation

of special stains is that the methods are empirical and non-specific, with considerable overlap between the natures of substances demonstrated by individual reagents. For instance, the periodic acid-Schiff stain is a multi-purpose stain used to demonstrate glycogen, basic mucins, fungal capsules, basement membrane and the granular cytoplasmic inclusions that are characteristic in the liver of patients with alpha-1-antitrypsin deficiency.

Table 2.

List of selected immunohistochemical stains employed in Diagnostic Histopathology

Diagnostic applications	Immunohistochemical stains
1. Identification of organisms	Monoclonal antibodies are available for a wide variety of bacterial, viral, fungal, protozoan and other organisms
2. Tumour classification	
Haematolymphoid neoplasms	CD45 positive, B cell markers (CD20, CD79a), B cell markers (CD3, CD5)
Neuroendocrine neoplasms	Neuron specific enolase, Chromogranin, Synaptophysin-positive (choose one marker)
Small blue cell tumours	Selected lymphoid, neuroendocrine, epithelial (e.g. pan-cytokeratin) and connective tissue (e.g. Myo-D1 for rhabdomyosarcoma) markers
Melanoma	S100, Melan A
3. Tumour prognostication	
Proliferative markers	Ki67, PCNA
Breast cancer	ER, PR, HER2

3. *Immunohistochemical stains*

Immunohistochemistry has expanded the vista of diagnostic histopathology globally, since an increasing variety of antibody reagents are now commercially available. Immunohistochemical staining can be applied to formalin-fixed paraffin-embedded tissues, to cytology samples, and even to electron microscopy, and is therefore an extremely versatile technique. Immunohistochemical stains involve the use of antibodies directed against specific tissue antigens or constituents. Immunohistochemistry remains the most widely used investigative tool for the identification and localisation of cellular antigens and gene products, in spite of the emergence of newer and more specific methods of detecting gene products and genes[9]. Just as for histochemical stains, the histopathologist should apply the use of immunohistochemistry judiciously. Good clinicopathological correlation of patient age, sex, site of biopsy, clinical features and results of ancillary investigations with haematoxylin-

eosin appearances is crucial to guide the selection of an appropriate panel of antibodies, particularly in a resource-poor tropical setting. Applications of immunohistochemistry include diagnosis of specific viral, bacterial, fungal, or protozoan infections; categorisation of undifferentiated malignant neoplasms; and tumour prognostication (Table 2). In the case for example of haematolymphoid malignancies, immunohistochemistry facilitates diagnostic decisions such as whether a lymphoid proliferation is benign or malignant; if malignant, whether it is a lymphoma; if a lymphoma, whether it is of Hodgkin or non-Hodgkin type; and then what precise type of Hodgkin or non-Hodgkin lymphoma[10]. Detection of antigen receptors in breast cancer cells is of prognostic and therapeutic value because oestrogen receptor positive cancers are susceptible to anti-oestrogen therapy and HER2 positive neoplasms are responsive to Trastuzumab and combination chemotherapy.

There are considerable challenges in establishing immunohistochemistry facilities in the tropics, not least of which is the cost of reagents. Other challenges include appropriate storage of antibodies and other reagents (buffers, antibody label, etc.) due to erratic electricity supply, the limited lifespan of antibodies, and the strict necessity for appropriate positive controls. Because of these difficulties, several clinicians and pathologists would rather opt to send their slides or blocks to diagnostic centres in Europe or North America for immunophenotyping than take on the challenge of establishing a local diagnostic service[11].

At the University College Hospital (UCH), Ibadan, Nigeria, the first diagnostic immunohistology service was established in 2004 at the Surgical Research Laboratory by Dr. Clement Adebamowo and collaborators[12]. Although it is acknowledged that there had been previous immunohistochemical studies from Ibadan[13,14,15] and other centres[16,17], Adebamowo's laboratory was the first Nigerian facility to offer large-scale, real time diagnostic services. Subsequently, a philanthropic body, the Aboderin Foundation, made available funds for lymphoma research, which permitted the launching of an immunohistochemistry service in the Department of Pathology, UCH, Ibadan. Incidentally, the Aboderin Foundation simultaneously made funds available for support of a similar service in the Lagos University Teaching Hospital, Idi-Araba, Lagos, Nigeria. The management of UCH subsequently invested in the expansion of immunohistochemistry services in the Department of Pathology, enabling application of a broad spectrum of antibody panels to common diagnostic problems encountered by the local hospital pathologists. Another important resource in Ibadan is located in the Breast Cancer Research Laboratory of the Institute of Advanced Medical Research and Training of the College of Medicine. This laboratory is supported by grants run by Professor Olufunmilayo Olopade in Chicago as the Chief Investigator, and Professor Grace Falade being the local institutional Principal Investigator in Ibadan.

It should be noted that immunohistochemistry facilities are now becoming more widely available in Nigeria. Centres such as the Abuja National Hospital, Obafemi Awolowo University Teaching Hospital Ile-Ife, Ahmadu Bello University Teaching Hospital, Zaria Bayero University Kano, the University of Benin Teaching Hospital, Benin-City University of Ilorin Teaching Hospital, Ilorin, Lagos University Teaching Hospital, Idi-Araba, Lagos[18,19] and a couple of privately run laboratories in the Lagos metropolis all have immunohistochemical facilities.

4. *Electron microscopy*

Electron microscopy is a procedure that involves the focussing of a beam of electrons by a powerful magnet on a piece of tissue. The deflected beams are then focussed by magnetic lenses on a cathode ray tube or photographic film to visualise the image. In light microscopy, the maximum resolution that can be achieved

is 250nm, but with electron microscopy, it approaches 1-2nm, which allows extremely high magnification, permitting the visualisation of ultrastructural features such as cytoplasmic organelles. The widespread application of cheaper and more rapid methods such as immunohistochemistry and molecular techniques have limited the use of electron microscopy to a few selected diagnostic scenarios. The use of electron microscopy also involves a delay interval of up to 2-3 weeks. However, there are important instances in which electron microscopy is the only technique that will render a definitive diagnosis (Table 3).

It is ironic that electron microscopic facilities were available in Ibadan, and a number of centres in Nigeria in the nineteen-seventies and early nineteen-eighties, but are no longer available in the twenty-first century[20]. The prohibitive cost of purchase and maintenance of this facility is the major drawback. Although there are a couple of functional electron microscopic facilities in the West African sub region, such as in Accra, Ghana, none of these is employed for routine diagnostic histopathology. This is a major drawback to scientific and clinical advances in the country. The most viable option for the utilisation of electron microscopy, which is also adopted by centres in developed countries, is rotational use of the facilities by several different units. For example, in the academic setting, an electron microscope purchased by a university could service research and diagnostic activities in the

Table 3.

List of selected uses of electron microscopy in Diagnostic Histopathology

Clinical application	Diagnostic feature
1. Renal pathology	
Minimal change disease	Loss of podocyte foot processes
Membranous glomerulonephrits	Immune complex deposition, (e.g.granular, linear, dense deposit disease)
2. Viral infections	Identification of specific virus
3. Neuropathology	
Mitochondrial myopathies	Abnormal mitochondrial inclusions
Metabolic myopathies	Identification of deposits (e.g. zebra bodies in Tay-Sachs and Niemann-Pick disease)
4. Tumour diagnosis	
Carcinomas	Cytoplasmic filaments, acinar differentiation, etc.
Neuroendocrine tumours	Dense core granules
Melanoma	Melanosomes

departments of human and veterinary anatomy and pathology, zoology and botany.

5. *Flow cytometry*

In flow cytometry, disaggregated cells in fluid suspension are made to pass, one cell at a time, through a laser beam that scatters light in different directions in amounts determined by the physicochemical characteristics of the individual cells. These reflected, refracted and unaltered light beams are detected by stationary detectors which transmit the summed and averaged information to a unit that generates histograms from which features such as cell size, cell shape, DNA content, and cell surface molecule expression may be generated for cell populations and sub populations. The Coulter counter routinely used in Haematology for determining full blood counts and differential counts is a very good example. Other uses of flow cytometry in haematology include determination of CD4 cell counts in HIV patients and the classification of haematological malignancies, particularly lymphomas and leukaemias.

In diagnostic histopathology, flow cytometry may be employed for tumour diagnosis. Normal tissues and benign lesions are generally diploid, when subjected to flow cytometric analysis. By contrast, malignant neoplasms are often aneuploid. To the best of our knowledge, apart from use in specialised HIV diagnostic laboratories, the use of flow cytometry in diagnostic histopathology or haematopathology is limited in sub-Saharan Africa.

6. *Molecular techniques*

Several types of molecular tests including *in situ* hybridisation, fluorescence *in situ* hybridisation polymerase chain reaction, and gene microarrays are available and can be applied not only to routine surgical biopsies, but also to core needle biopsies and fine needle aspirates[21].

(a.) In situ hybridisation

This technique is applied to the demonstration of specific nucleic acid sequences in tissue sections and cell preparations. *In situ* hybridisation localises target sequence to specific cells or tissue types. The target sequence of DNA is visualised by tagging with radioactive (S^{35}) or non-radioactive (peroxidase, biotin, digoxigenin) labelled probes.

(b.) Fluorescent in situ hybridisation (FISH)

This technique uses fluorochrome labelled nucleic acid probes to demonstrate specific DNA sequences and can also be applied to routinely processed tissues. Applications include demonstration of chromosomal translocations (e.g. *BCR-ABL* in chronic myelogenous leukaemia) and gene amplifications (e.g. *HER2* and *MYCN* amplifications in breast carcinomas and neuroblastomas, respectively)[22].

(c.) Polymerase chain reaction (PCR)

This technique uses a heat stable DNA polymerase (such as Taq polymerase) to amplify selected DNA sequences exponentially. Amounts of DNA as small as 35 kb can be amplified one billion-fold after just 30 cycles of PCR. Each PCR cycle involves DNA denaturation at 90^0C, primer annealing at 55^0C and polymerase extension at 72^0C (the last being the optimum temperature for Taq polymerase activity). There are several variations on the basic method, one of which (Hybrid capture) has been used for HPV screening of cervical smears. The advantages of PCR over *in situ* hybridisation include its high sensitivity and extreme rapidity, with the use of automation. Conventional PCR yields results within two to four hours, while real-time PCR is as rapid as 30 minutes[22].

(d.) Nucleic acid microarray technology (Gene profiling)

Tissue microarrays are slides of cores of tissues arranged in an organised grid. These cores of tissues are removed from donor blocks of tissue, and deposited in a recipient block. This recipient block is then sectioned to produce the tissue microarray that is used in the final experiment[23]. This technique may be applied to special stains, immunohistochemistry and

the molecular techniques earlier described. The process of measuring the expression of thousands of genes simultaneously in a given tissue sample generates an expression profile. The basic principle is hybridisation based[24]. Microarray technology can be applied to DNA sequences (gene microarrays), and proteins (protein microarrays)[24].

Molecular techniques are undoubtedly the gold standard for definition of specific disease entities. However, these are still largely research methodologies, even in the most developed countries and remain to be incorporated into routine diagnostic practice. Major drawbacks include their high cost and the need for fresh or frozen tissues[25]. Inspite of these limitations, an increasing number of laboratories in North America and Europe, particularly in the histopathology sub specialties are incorporating cutting edge molecular technology into their real-time diagnostic armamentarium.

Concluding Remarks

As was noted over twenty years ago, histopathology, even more so than other branches of laboratory medicine, has a pivotal role in tropical medicine. Histopathology is essential in the diagnosis of infectious diseases, the definition of new pathological entities, collection of data on morbidity and mortality, and support of basic advancement and research on tropical diseases[26].

There is no doubt that collaborations between home-based pathologists in tropical developing countries and their colleagues based in developed countries have immense potential for enhancement of diagnostic services in the resource-poor settings of tropical developing countries. One of these is Pathologists Overseas, an initiative of Heinz Hoenecke, a German histopathologist, which presently comprises over 50 volunteer retired and practicing pathologists from the USA, Canada and Australia. Pathologists Overseas has started laboratories and teaching programmes in East and West Africa; and is active in Nepal, St. Lucia and Bhutan[27]. An offshoot of this programme is the planned re-establishment of sustained surgical pathology services at the University of Kumasi Teaching Hospital, through collaboration with the University of North Norway[28].

Yet another multi-national initiative is the Friends of Africa programme of the United States and Canadian Academy of Pathologists (http://www.uscap.org) and the International Academy of Pathology (IAP). There are South African, West African and North African Divisions of the IAP, with sister divisions in all six continents. International faculty have contributed immensely to loco-regional conferences of these divisions. Extensive teaching and clinical material has also been made available on the Internet free of charge to pathologists from developing countries.

The shortage of indigenous pathologists needs to be addressed internally by national policies that enable an increased output of physicians. This is no mean task since there is a paucity of medical schools in East and West Africa, with only about 20 medical schools in Nigeria, the largest number in the sub region. There is also need to recruit brilliant medical students into Laboratory Medicine, by maintaining high clinical and teaching standards and thereby serving as role models. A recent study from Jos, Nigeria has shown that almost a fifth of graduating medical students considered laboratory medicine to be a preferred choice of future specialisation[29]. However, it would not do to lose the majority of these young aspirants to the rarefied and more lucrative climes of hospitals and laboratories abroad, whereas there is still an acute shortage of pathologists in the country. Thus, efforts have to be implemented by professional regional, national and international bodies (such as the various regional postgraduate medical colleges, national medical associations and professional pathology groups) to ensure that their individual members and accredited training centres are of internationally acceptable standard.

National and state governments should be compelled to inject massive doses of financial, manpower and infrastructural resources into the health care system by all that have the privilege of representing their professional colleagues in the political and administrative arenas. It is important to note that there has to be an even development not only of laboratory physicians, but also of laboratory scientists, in order for the full impact of this investment to be realised.

It should however be recognised that there is a limit to the extent of government spending on health care delivery, even in developed countries. Government funded hospital laboratories, especially so in resource-limited settings, need to be financially accountable, seek cost saving alternatives, and budget according to volume of clinical and research activity in the hospital, to minimise wastage of limited resources. Above all else, these public funded facilities need to strive to maintain a level of academic and professional excellence that sets them apart from privately run laboratories[30].

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