

The use of special stains in liver biopsy interpretation: Implications for the management of liver disease in Nigeria

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Abstract

Context: The evaluation of a liver biopsy requires the use of stains other than routine hematoxylin and eosin (H and E) to highlight many important features.

Aims: Most Nigerian Histopathology Departments do not routinely perform special stains (personal communication by authors). This study aims to re-evaluate a set of liver biopsies which has been diagnosed solely on H and E stains by performing a standard set of special stains on them.

Settings and Design: This is a retrospective analysis.

Materials and Methods: The formalin fixed paraffin embedded blocks of liver biopsies reported in two histopathology laboratories between 2008 and 2013 were retrieved. These were stained with H and E and the following standard special stains for liver tissue histology – Perl's Prussian blue, reticulin, Sirius red, Shikata orcein, and periodic acid-Schiff with diastase. The stained slides were re-analyzed.

Statistical Analysis Used: No formal statistical analysis was performed, but results are summarized and tabulated by summary statistics, where appropriate.

Results: Seventy-four liver biopsy paraffin blocks were received in the laboratories. Fifty-three (71.6%) were suitable for analysis out of which 51 (68.9%) had their clinical details retrievable. In 29 cases (56.9%), Perl's stain was positive for iron pigment within the hepatocytes with 17 (58.6%) of these being Grade 1, 7 (24.1%) Grade 2, and 5 (17.2%) Grade 3. Shikata orcein revealed hepatitis B viral surface antigen in 15 (29.4%) of the cases while copper-associated protein was demonstrable in 6 (11.8%) of the cases. The discovery of stainable iron implies some degree of disturbance of iron metabolism, and a Grade 3 stainable iron requires investigation for genetic hemochromatosis. The demonstration of copper-associated proteins suggests biliary disease in a noncirrhotic liver which also requires further investigation.

Conclusion: This study confirms the need to routinely perform special stains in reporting liver biopsies to fully investigate and manage patients and their relatives.

Key words: Biliary disease, iron overload, liver biopsy, special stains

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Introduction

Ever since Erlich performed the first liver aspirate in 1883 and the first percutaneous liver biopsy was performed in the 1920s, liver biopsy has become established as the gold standard for the diagnosis and staging of liver diseases.^[1]

Previous studies from Nigeria show that the most common histologic diagnoses of biopsies from patients with chronic liver disease are mainly chronic hepatitis, hepatocellular carcinoma (HCC), cirrhosis, and metastatic carcinoma with varying proportions from hospital to hospital.^[2] Alcoholic liver disease and nonalcoholic fatty liver disease are quite rarely reported in the studies.^[2]

Liver biopsies are useful in diagnosing the disease, determining the severity of disease and the suitability of patients for treatment. The severity of hepatitis and cirrhosis is determined based on the degree of inflammation, and fibrosis present in the specimen.

The evaluation of a liver biopsy requires the use of stains other than routine hematoxylin and eosin (H and E) to highlight the architecture, degree of fibrosis, and some important features including viral inclusions and pigments. The role of special stains, such as periodic acid-Schiff with diastase (PASD), Perl's Prussian blue, Shikata orcein, reticulin and Sirius red/trichrome stains, in evaluating liver biopsies is crucial and cannot be over-emphasized. It is even recommended that these stains should be re-classified as "routine" and not "special" since they are essential for liver biopsy interpretation.^[3] Inter- and intra-observer variability in the H and E assessment of fibrosis in liver biopsies is another good reason for the use of a collagen stain such as Sirius red or trichrome in reporting biopsies.^[4]

In most Departments of Histopathology in Nigeria, these stains are not routinely performed on liver biopsies (personal communication by authors), and diagnosis is made purely based on the H and E. This study aims to re-evaluate a set of liver biopsies which has been diagnosed solely on H and E stains by performing a standard set of special stains on them and see if there are any differences in the diagnoses.

Materials and Methods

The formalin fixed paraffin embedded (FFPE) blocks of liver biopsies reported in two large histopathology laboratories in Lagos between 2008 and 2013 were retrieved. These have been reported by many different pathologists using H and E staining only and various grading and staging systems for chronic hepatitis including Scheuer, Metavir, and Ishak had been used.

Fresh histology slides were made and stained with H and E and with the following special stains: PASD, Shikata orcein, reticulin, Perl's Prussian blue, and Sirius red. The slides were then re-examined by two pathologists who were blinded to the original diagnoses and clinical details of each patient to avoid bias. Comparisons were then made between the original H and E diagnosis, and the diagnosis reached after examination with special stains. Staging and grading of fibrosis and inflammation were done with the Ishak scoring system.

Exempted from the study were slides with too little tissue left in the paraffin blocks. Biopsies with <6 portal tracts were considered inadequate but reported on in the presence of significant findings. In assessing the number of portal tracts, we counted them on H and E and then used PASD stain to corroborate the count. The UK Royal College of Pathologists (RCPATH) guidelines suggests a minimum of six portal tracts and a length of 1 cm per biopsy.^[5]

The "special" stains that were used in this study and their purposes are as follows:

- Sirius red: It helps in staging fibrosis by staining collagen fibers red. Sirius red stains type 1 and 3 collagen found in the portal tract and can be used to correlate portal tract count done on H and E. It has also been found to be more useful than trichrome in computer-assisted fibrosis imaging techniques and correlates better with serum markers of fibrosis.^[4,6] It also stains bile green and amyloid protein red
- Shikata orcein: This demonstrates hepatitis B surface antigen (HBsAg) inclusions and copper-associated protein within hepatocytes. It can also be used to date fibrosis based on the presence of elastic fibers which are laid down in old fibrous tissue
- PASD: This demonstrates α -1-antitrypsin globules within the hepatocytes and ceroid bodies (residual bodies) within Kupffer cells. It also outlines the basement membranes of biliary ducts
- Perl's Prussian blue stain: This identifies in ferric iron (Fe) pigment. In our study, the iron deposits in hepatocytes were graded from grades 0 to 4 in increasing order of severity with 0 being absent and 4 representing intensely clumped iron within the cells. Where possible, iron found predominantly in Kupffer cells was differentiated from that predominantly within the hepatocytes
- Reticulin: This stains the normal reticulin framework of the liver and, therefore, highlights areas of reticulin collapse and reticulin deficiency.

Results

Seventy-four liver biopsy paraffin blocks were received in the laboratories between January 2008 and December

2013. Fifty-three paraffin blocks (71.6%) were suitable for analysis out of which, only 51 (68.9%) had their clinical details retrievable.

The ages of the patients ranged from 33 days to 75 years with an average age of 59 years. There were 41 males (80.4%) and 10 females (19.6%). Of the 51 cases analyzed, 30 were judged to be adequate with six or more portal tracts. Twenty-one had fewer portal tracts and were potentially inadequate for re-analysis; however, in nine of these, there were significant findings worth commenting upon.

Table 1 shows a summary of the diagnoses made before and after special stains were used.

Table 1: Summary of diagnosis before and after special stains			
Diagnosis after special stains	n (%)	Initial diagnosis	n (%)
Chronic (viral) hepatitis	27 (53)	Chronic (viral) hepatitis	34 (66.7)
Cirrhosis	6 (11.8)	Cirrhosis	5 (9.8)
Malignant	3 (5.9)	Malignant	3 (5.9)
Suspected hydatid disease	2 (3.9)	Reactive	2 (3.9)
Extramedullary hematopoiesis	2 (3.9)	Sickler with acute liver cell injury and sinusoidal congestion	1 (2)
Clinicopathological correlation required	2 (3.9)	Fatty change	1 (2)
Vascular etiology	2 (3.9)	Sinusoidal lymphocytosis	1 (2)
Normal	1 (2)	Normal	1 (2)
Primary sclerosing cholangitis	1 (2)	Sclerosing cholangitis	1 (2)
Inadequate	10 (19.6)	Biliary atresia	1 (2)

Note that there may be overlapping diagnoses

The age and sex of eight selected cases with significant discrepancies between the original and reviewed diagnoses and the clinical information provided on the request cards are displayed in Table 2.

The use of special stains also revealed important results including pigments and viral inclusions that were not part of the original H and E diagnoses. In 29 cases (56.9%), Perl's stain was positive for iron pigment predominantly in the hepatocytes with 17 (58.6%) being Grade 1, 7 (24.1%) Grade 2, and 5 (17.2%) Grade 3. In four cases, the iron pigments were predominantly in Kupffer cells.

Shikata orcein revealed HBsAg in 15 (29.4%) of the cases while copper-associated proteins were positive in 6 (11.8%)

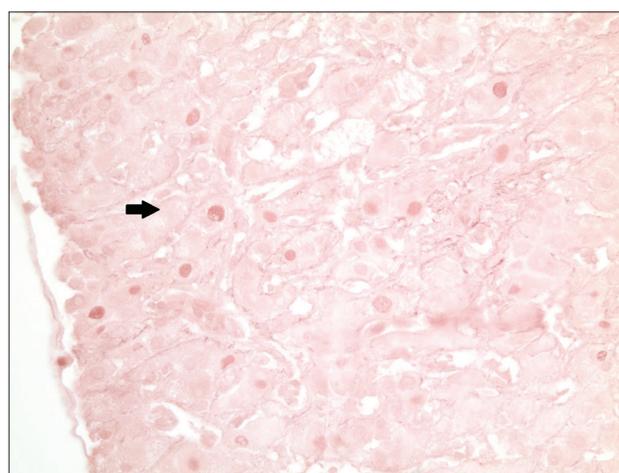


Figure 1: Positive Shikata stain for hepatitis B surface antigen (Shikata, ×200)

Table 2: Clinical details, initial diagnosis and reviewed diagnosis of selected cases				
Age	Sex	Clinical hx	Original diagnosis	Reviewed diagnosis
48	Male	HCC	Mild chronic hepatitis, inactive	Suspicious for extramedullary hematopoiesis, Suspicious for sepsis Iron in Kupffer cells
75	Male	Suspicious for PLCC, hepatic mass and CT-scan features of HCC	Normal	Inadequate biopsy. Suspicious for peliosis hepatitis Grade 3 iron
34	Male	Chronic HBV infection with intrahepatic mass lesion seen on ultrasound*	Chronic hepatitis with moderate necroinflammation, moderate fibrosis (G2S2) probably due to HCV infection	Chronic HBV hepatitis with significant necroinflammation and fibrosis (G6S5), Grade 2 iron. Active steatohepatitis
25	Male	Chronic HBV infection with elevated liver enzymes	Chronic hepatitis with Scheuer grade 1/4 and stage 1/4	Normal biopsy
41	Male	Infiltrative liver disease unknown cause	Fatty change	Zone 3 vascular pathology (veno-occlusive disease). Suspicious for heart failure. Grade 1 iron
35	Female	HBV, elevated LFTs	Reactive	chronic hepatitis G2S0, Grade 1 iron
36	Male	Chronic HBV infection with high viremia and minimally elevated LFT	Chronic hepatitis with moderate necroinflammation and portal-portal fibrosis (scheuer activity grade 3/4 and stage 2/4)	Suspicious for hydatid cyst B11/634
47	Female	Chronic disease with severe anemia of 5 months	Chronic hepatitis, mild necroinflammation, minimal fibrosis	Extramedullary hematopoiesis. Iron in Kupffer cells

HBV=Hepatitis B virus; LFT=Liver function test; HCC=Hepatocellular carcinoma; CT=Computed tomography; PLCC=Primary liver cell carcinoma

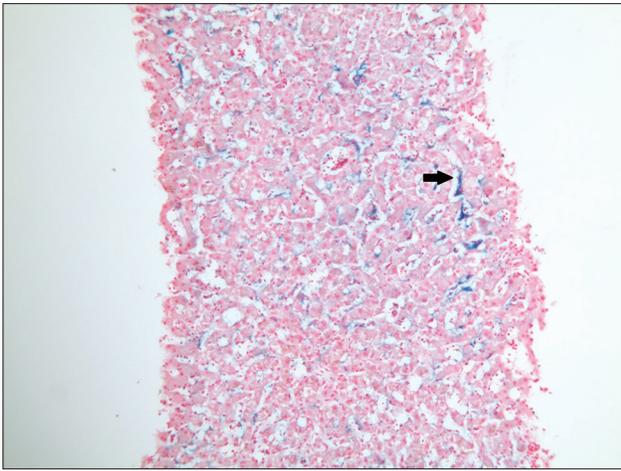


Figure 2: Same sample as A, arrow shows positive Perl reaction within the Kupffer cells. (Perls, $\times 100$)

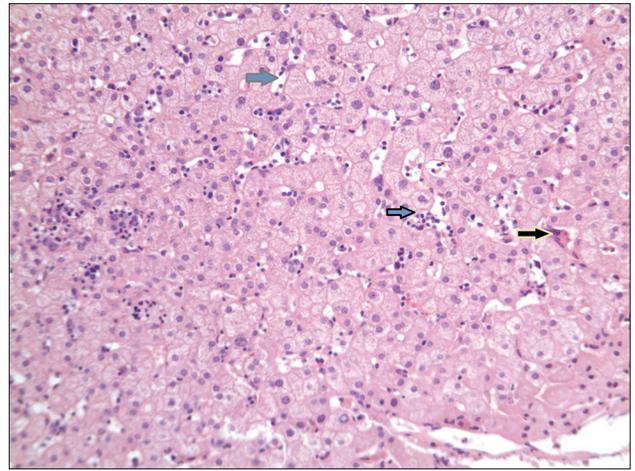


Figure 3: Arrow shows hematopoietic cells in the sinuses of the liver. Black arrow indicates the hemopoietic cells; the yellow outlined arrow indicates a megakaryocyte (H and E, $\times 400$)

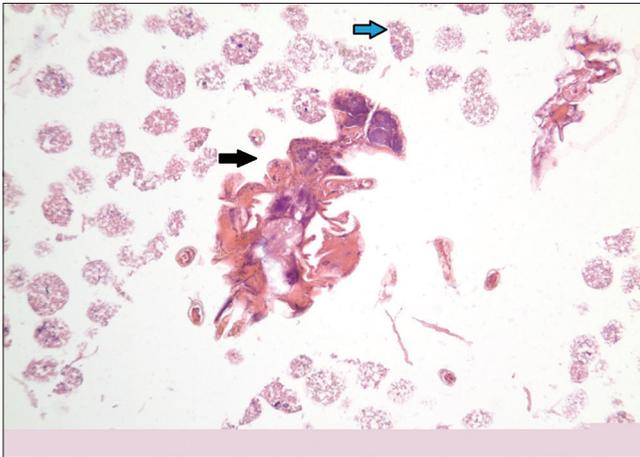


Figure 4: Hydatid cyst (black arrow) and daughter cysts (blue arrow)

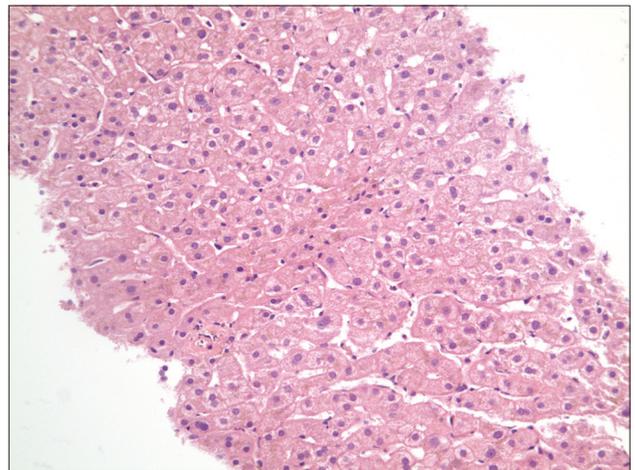


Figure 5: Apparently "normal" histology (H and E, $\times 100$)

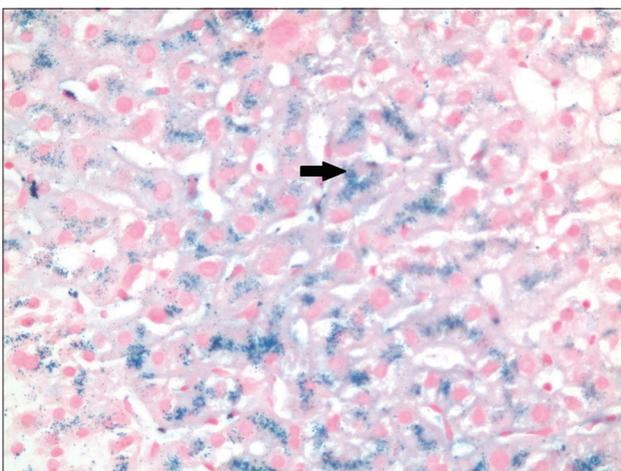


Figure 6: Same sample as in E. Arrow shows Grade 3 iron in hepatocytes (Perls, $\times 40$)

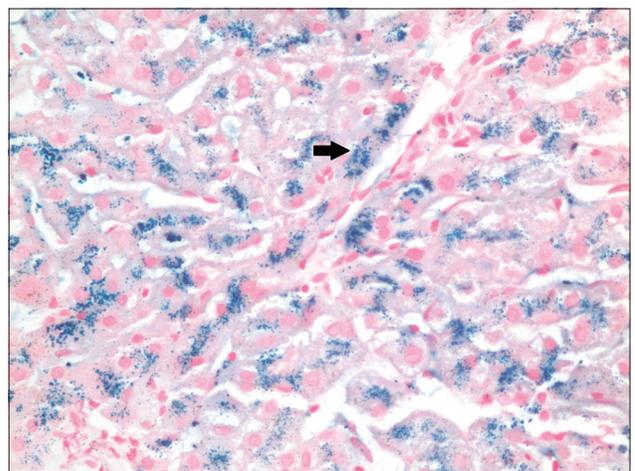


Figure 7: Same sample as in E. Arrow shows Grade 3 iron in hepatocytes (Perls, $\times 400$)

of the cases. Figures 1-7 show selected photomicrographs of our findings.

Discussion

The male to female ratio in our study was 4:1. This is similar to the ratio seen in other studies on liver biopsies in the Nigerian literature, for example, Abdulkareem *et al.* reported a ratio of 3:1,^[2] while Ndububa *et al.* reported a ratio of 1.5:1.^[7]

The limitations of this method of study include inadequate quantity of tissue left in the paraffin blocks due to the effect of cutting fresh sections after trimming. This limits interpretation and may account for nine inadequate sections. However, these inadequacies should not deter from reporting the cases as the use of special stains can still highlight important features such as iron, copper, or HBsAg. Another limitation is that this is a retrospective work on a highly selected group of patients and, therefore, generalization cannot be made of the results. We reviewed them blindly to minimize bias, but this limits clinicopathological correlation which is very important in liver biopsy interpretation.

The prevalent hepatic finding in our study being chronic hepatitis is also as described in other African literature.^[2,7,8] We found that chronic hepatitis from hepatitis B virus (HBV) infection was more common than that from hepatitis C virus (HCV) in this series. This is in keeping with serologic studies done in various parts of Nigeria on patients presenting with liver disease showing HBV as more prevalent than HCV.^[9] This also points to the leading role of HBV in the causation of liver disease in Nigeria.^[9,10]

In this study, the importance and indispensability of using special stains in liver biopsy interpretation were highlighted in the number of biopsies that were positive for iron pigment, copper-associated proteins, and for HBsAg viral inclusions which are not visible on routine H and E examinations.

In this study, a good proportion of the biopsies (56.9%) were positive for iron pigment mostly within the hepatocytes. All the patients with Grade 3 (100%) and six of Grade 2 iron (85.7%) were male. This agrees with the male predominance found by Barton *et al.* in their study on hepatic iron deposition in Africans.^[11]

The deposition of iron in the Kupffer cells can be seen in hemosiderosis which may result from repeated blood transfusions in sickle cell disease patients as was seen in one of the cases. Studies have shown that the presence of iron as hemosiderin granules in liver biopsies is of important clinical significance. In patients with sickle cell disease or β -thalassemia receiving red blood cell transfusions for a long period, a precise knowledge of the liver iron

concentration (LIC) is essential for treatment.^[12] The LIC can be assessed from FFPE liver biopsies.

The demonstration of stainable iron in liver tissue has important clinical associations and may be the sole cause of liver disease or may be contributing to liver damage in the presence of another known disease. For example, Grade 3 iron deposition in a male patient raises a strong suggestion of genetic hemochromatosis which requires further investigations. Missense mutations of the hemojuvelin gene HJV on Ch1q^[13] or the erythroid-specific 5-aminolevulinate synthase gene ALAS2 on ChX^[14,15] has been reported in African Americans with predominant hepatocyte iron deposition.

Furthermore, the association of hemosiderosis with the inheritance of the Q248H missense mutation of the ferroportin 1 gene (FPN1) has been reported in African Americans.^[16] Barton *et al.* found that 2 of 13 (15.4%) African American iron-overload index patients and 2 of 39 (5.1%) African American control subjects who reside in central Alabama were heterozygous for *FPN1 Q248H*.^[16] However, performing DNA analyses to detect mutation of iron-associated genes is beyond the scope of this work.

It has been said that a greater proportion of African Americans than persons of other races responds to chronic hepatitis C infection with an increase in iron stores, after adjustment for age, alcohol intake, gender, menopausal status, education, body mass index, and poverty index.^[17] Majority of the present subjects who had heavy iron staining had hepatic inflammation. It is possible that some of these subjects had viral hepatitis C or significant alcohol although this is unproven.

Ioannou *et al.* described significantly elevated markers of iron overload in the serum of subjects who consumed more than two alcoholic drinks/day.^[18] We did not have significant information on the request forms to include alcohol as a significant factor in the iron deposition found in these patients.

Iron deposition in the Kupffer cells is said to worsen inflammation and fibrosis in patients with nonalcoholic fatty liver disease.^[19] In a study involving patients with chronic active hepatitis C,^[20] liver iron accumulation was significantly associated with increased histological activity and cirrhosis while a study involving patients with HBV infection^[21] showed that iron deposition was associated with higher activity and fibrosis. From these studies, it can be implied that iron deposition in a virus-infected liver may lead to accelerated decompensation. Further studies^[22] point to liver iron deposition as being associated with accelerated decompensation and decreased survival in patients with cirrhosis. The synergistic effects of HBV or HCV infection, alcohol, and iron deposition may lead to

accelerated development of fibrosis and cirrhosis. This shows the importance of detecting iron deposition in liver biopsies as its presence implies the need for closer follow-up of the patient. Iron also independently increases the risk of HCC and may act synergistically with other causes in malignant transformation of hepatocytes

The use of Shikata orcein serves a triple function in the assessment of liver biopsies as previously mentioned. With the advent of immunostaining for assessment of HBsAg and HBeAg, the high cost and nonavailability of these markers leave low resource laboratories with no choice but to use Shikata orcein. Through its use, we were able to identify 15 HBsAg positive cases (without any clinical information) and this makes for good clinicopathological correlation. It can also be useful in the exceptional case where serum markers for HBV have not been done. Weak positive staining of hepatic parenchyma should be evaluated with caution when serologic tests for HBsAg are negative. It may warrant repeated serologic testing.^[23]

Although the concentration of copper-associated protein cannot be quantitatively determined by orcein staining, positive hepatocytes give a useful clue to the possible presence of biliary disease (such as primary biliary cirrhosis and primary sclerosing cholangitis) and metabolic disorder of copper metabolism (Wilson's disease) in a noncirrhotic liver. Increased copper concentration in hepatocytes has been described in primary biliary cirrhosis.^[24] The demonstration of copper-associated protein is therefore a useful re-assuring result in evaluating biliary disease as demonstrated in one of the cases reviewed.

PASD identified ceroid bodies in the Kupffer cells and this implies ongoing or recent hepatocyte damage and this was positive in eight cases. No case of alpha-1-antitrypsin deficiency was detected, but larger population studies may find that this condition is not as rare among Nigerians as we are wont to believe.

Most of the discrepancies seen between the original and reviewed diagnosis were related to the grading of inflammation and staging of fibrosis and the nonreporting of liver pigments. Discrepancies in fibrosis staging accounted for 58% of inter-observer variability seen by Colling *et al.* in an assessment of discrepancy in liver biopsy reporting between their specialist and other general practice centers.^[25] Although the staging of fibrosis in biopsies is said to be prone to inter-observer variability even among "experts,"^[25] the use of such terms as "mild," "moderate," "severe" as grades for fibrosis should be avoided as they only serve to deepen variability among pathologists. However, because the clinicians appreciate such terminologies,^[24] we recommend that these terms be used only after a fibrosis score has been assigned in the pathology report. It is worthy

of note that in most of our subjects with discrepancy in staging, the fibrosis was understaged. Moreover, it is best for a unit to decide on a staging and grading system in discussion with the clinicians and such should be used consistently.

More serious discrepancies most commonly result from a lack of expertise^[24] and are likely to have major clinical impact in the management of patients in the absence of proper follow-up. The development of expertise in liver biopsy reporting requires considerable experience. Maintenance of this expertise requires continuous exposure to a minimum number of cases each year. Measures that have been proposed by the UK RCPATH^[25] which can be adapted to our environment include having a named team lead for liver pathology in each center, external quality assessment participation, and a minimum annual exposure to liver biopsy cases (20 slides per pathologist suggested). Other suggestions are consensus reporting for liver pathologies and external audit of a sample of cases (about 10%). Centers without sufficient numbers of liver biopsies can send their FFPE samples to better-equipped centers (with clear criteria for referral).^[24]

Conclusion

The use of special stains in liver biopsy interpretation has gone beyond being an adjunct to being a necessity. In our study, it was remarkably useful in counting portal tracts, highlighting basement membranes, HBsAg inclusions, copper and ferric iron pigments, ceroid bodies, and in the reproducible staging of fibrosis. It is easy to use and affordable. We suggest that liver biopsies should not be reported without the use of special stains as this does not help patient management nor does it improve pathology practice.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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