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# Histochemical analysis (special stains) in the detection and identification of histologic and histopathologic changes in the liver tissue of neonates

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ABSTRACT

**Background:** Perinatal morbidity and mortality continue to be a major challenge in the developing world. The histochemical approach to diagnostic pathology is based on correlating the presence or absence of morphological changes with any biochemical lesion. The objective of this study is to demonstrate the relevance of special stains on the effective diagnosis of neonatal morbidity associated with liver damage, disease, anomalies or congenital conditions.

**Methods:** Forty-six (46) Formalin Fixed Paraffin Embedded (FFPE) blocks of autopsied neonatal liver tissues were retrieved from the Department of Pathology University of Calabar Teaching Hospital, Calabar. Fresh histology sections were made and stained with H&E and with the following histochemical staining techniques (special stains) Periodic Acid Schiff's (PAS), Orcien, Reticulin, Perl's Prussian blue and Masson Trichrome.

**Results:** Perl's Prussian staining was positive in significant amount (26.09%) indicating iron deposition which could be due to congenital malarial or congenital haemolytic disorder. Orcein staining was also positive in 21.74% of the samples displaying features of congenital hepatitis. PAS was positively stained in all the sections demonstrating bile duct basement membrane damage in biliary diseases. Masson Tricrome was positive in 17.39%, indicating the degree of fibrosis in the liver. Reticulin staining was positive in all the sections indicating hepatic damage.

**Conclusion:** The application of histochemical techniques to laboratory investigations of tissue samples can yield considerable information. Histochemical analysis of the liver tissue can give insight into the liver function and can pinpoint and localize a wide variety of carbohydrates, proteins, lipids and enzymic activities.

**Keywords:** histochemistry, special stain, neonate, liver

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## INTRODUCTION

The liver, the largest gland in the body, is critical to many key metabolic functions [1]. It also plays an important role in removing the waste products of metabolism particularly ammonia and in detoxifying drugs and other substances such as endogenous hormones and steroid compounds. In addition, the liver plays a major role in the production of clotting factors, plasma proteins, bile salts, and bilirubin [2].

Many newborns have hepatic dysfunctional symptoms such as hyperbilirubinemia, hepatomegaly, or increased liver enzyme levels [3]. These frequently develop after the administration of medications or parenteral nourishment to address other issues, or as a side effect of systemic illnesses such as sepsis or hypoxic damage [4]. Although it is uncommon, primary liver disease can develop in newborns. If it happens, it must be quickly diagnosed and treated to avoid needless sequelae [5].

Autopsy has contributed to medical care since the advent of modern medicine, by establishing the cause of death, assisting in determining the manner of death, comparing antemortem and postmortem findings, producing vital statistics, or monitoring public health [6]. In the paediatric population, autopsies may also guide genetic counselling and assist grieving families [7]. In providing a final and correct diagnosis, the autopsy serves to reassure relatives and alleviate feelings of guilt or blame relating to the death of the deceased [8, 9]. Despite the importance of autopsy in establishing a final diagnosis and providing advice, there has been a worldwide decline in the neonatal autopsy rate [10].

Special stains remain important tools for many pathologists, providing a powerful complement to immunohistochemistry, flow cytometry, in situ hybridization and other diagnostic technologies that ultimately define a patient's medical profile [11]. Many special stains are key to detecting and identifying pathogens, while others play an important role in diagnosing and monitoring cancer. Iron stains can indicate hemochromatosis or iron deficiency, the Masson's Trichrome stain demonstrates changes in collagen and muscle cells associated with cirrhosis, and the Periodic Acid-Schiff stain provides information about glycogen storage disease [12, 13]. The study aims to demonstrate the relevance of histochemical analysis (special stains) in the detection and identification of histologic and histopathologic changes in the liver tissue of neonates.

## MATERIALS AND METHODS

A total of 46 Formalin-Fixed Paraffin-Embedded (FFPE) blocks of autopsied neonatal liver tissues were retrieved from the Department of Pathology University of Calabar Teaching Hospital, Calabar. These have been reported by pathologists using Hematoxylin and Eosin staining only. Fresh histology slides were made and stained with H&E and with the following special stains: (Periodic Acid Schiff's) PAS, Orcien, Reticulin, Perl's Prussian blue and Masson Trichrome. The stained slides were then re-examined by two pathologists who were blinded to the original diagnoses and clinical details of each to avoid bias.

Information regarding the time of admission, gestational age, age at admission, birth weight and age at death were retrieved from the records of UCTH. The principles, procedures and expected results from the histochemical staining techniques used for the study are presented in the appendix. The relevant clinical data and histologic and histochemical characteristics were analyzed using predictive analytical software, version 26 (IBM, SPSS Inc., Chicago, IL, USA).

## RESULTS

Forty-six (46) Formalin-Fixed Paraffin-Embedded (FFPE) blocks of autopsied neonatal liver tissue were retrieved from the Department of Pathology, University of Calabar Teaching Hospital, Calabar. These have been reported by pathologists using Haematoxylin and Eosin staining only. Fresh histology slides were made and stained with H&E and with the following special stains: PAS, Orcien, Reticulin, Perl's Prussian blue and Masson Trichrome. The slides were then re-examined by two pathologists who were blinded to the original diagnoses and clinical details of each to avoid bias.

According to the information retrieved from the record of UCTH, the neonates were admitted at the first few hours of life as a result of complications of labour to the sixth day of life. Age at admission, age at death and gestational age at birth are presented in tables 1-4 respectively. There were 29 males (63.04%) and 17 females (36.96%). The birth weight of the neonates varies from 1 kilogram to 4 kg, 5(10.87%) presented with very Low Birth Weight (VLBW), 15(32.61%) presented with Low Birth Weight (LBW), 22(47.83%) majority of the neonates were with Normal Birth Weight (NBW) as presented table 5. The organ (liver) weights of neonates are presented in table 6.

The clinical presentations of the neonates are; cord neck 8(17.39%), fever 4(8.70%), low birth weight 4(8.70%), poor suck 4(8.70%), poor cry 8(17.39%), prematurity 7(15.22%) postdate were presented in 9(19.57%) and spasm 2(4.35%) respectively as shown in table 6.

The cause of death of the neonate with the aid of anatomical findings and routine histological staining with Haematoxylin and Eosin were Birth trauma (2.17%), Hyaline membrane diseases (4.35%), congenital heart disease (19.57%), Kernicterus (2.17%), Neonatal sepsis (39.13%), Prematurity (17.39%), Severe Birth Asphyxia (8.69%). The findings in the Liver with Haematoxylin and Eosin stains were Chronic Venous congestion (65.22%), Fatty change (6.52%) and Hepatic cyst (4.38%). The anatomical findings and routine histological H&E staining findings are presented in tables 7-9 respectively. 8(17.39%) of the liver samples were positive for Masson Trichrome stains and the remaining 38(76.47%) samples were negative for Masson Trichrome stains.

10(21.74%) of the neonatal liver samples were positive for Orcein stains and the remaining 36(78.26%) samples were negative for Orcein stains. 12(26.09%) of the Liver samples were positive for Perl's Prussian blue stain and 34(73.91%) were negative for Perl's stain. All samples fourth-six samples were positive for PAS (100%) and Reticulin stains (100%) respectively. Photomicrographs of the various special stains are presented in figure 1 through figure 12.

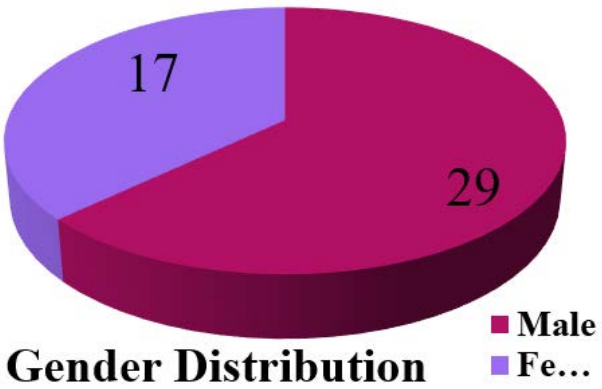


**Fig. 1.** The sex of the neonates

**Tab. 1.** Age distribution of neonates on admission to the hospital

| No. of Days | Frequency | Percentage (%) |
|---|---|---|
| 0 | 21 | 45.65 |
| 1 | 12 | 26.09 |
| 3 | 9 | 19.57 |
| 6 | 4 | 8.69 |

**Tab. 2.** Age at death of the neonates

| No. of Days | Frequency | Percentage (%) |
|---|---|---|
| 1 | 18 | 39.13 |
| 2 | 16 | 34.78 |
| 4 | 5 | 10.87 |
| 8 | 3 | 6.52 |
| 9 | 1 | 2.17 |
| 10 | 2 | 4.34 |
| 21 | 1 | 2.17 |

The age ranges from 1 day to 21 days

**Tab. 3.** Gestational age of the neonates

| No. of Weeks | Frequency | Percentage (%) |
|---|---|---|
| 29 | 1 | 2.17 |
| 35 | 13 | 28.26 |
| 36 | 9 | 19.57 |
| 37 | 14 | 30.43 |
| 38 | 5 | 10.87 |
| 41 | 3 | 6.52 |
| 42 | 1 | 2.17 |

Majority of the neonates were full term

**Tab. 4.** Body weight at birth of neonates

| Approximate Weight in Kilogram (Kg) | Frequency | Percentage (%) |
|---|---|---|
| <1(ELBW) | 0 | 0 |
| 1-1.49(VLBW) | 5 | 10.87 |
| 1.5-2.49(LBW) | 15 | 32.61 |
| 2.5-3.49(NBW) | 26 | 56.52 |
| >4(HBW) | 0 | 0 |

ELBW – Extremely Low Birth Weight, VLBW – Very Low Birth Weight, LBW – Low Birth Weight, NBW – Normal Birth Weight, HBW – High Birth Weight

**Tab. 5.** The organ (liver) weight of the neonate

| Liver Weight (Grams) | Frequency | Percentage (%) |
|---|---|---|
| 48 | 4 | 8.7 |
| 61 | 1 | 2.17 |
| 62 | 5 | 10.87 |
| 65 | 3 | 6.52 |
| 73 | 2 | 4.35 |
| 84 | 3 | 6.52 |
| 94 | 4 | 8.7 |
| 96 | 6 | 13.04 |
| 101 | 2 | 4.35 |
| 107 | 1 | 2.17 |
| 120 | 3 | 6.52 |
| 121 | 3 | 6.52 |
| 173 | 1 | 2.17 |
| 197 | 4 | 8.7 |
| 199 | 1 | 2.17 |
| 250 | 2 | 4.35 |
| 277 | 1 | 2.17 |

**Tab. 6.** The Clinical Presentation of the Neonates

| Clinical Presentation | Frequency | Percentage (%) |
|---|---|---|
| Cord neck | 8 | 17.39 |
| Fever | 4 | 8.7 |
| LBW | 4 | 8.7 |
| Poor cry | 8 | 17.39 |
| Poor suck | 4 | 8.7 |
| Postdate | 9 | 19.57 |
| Prematurity | 7 | 15.22 |
| Spasm | 2 | 4.35 |

LBW – Low Birth Weight

**Tab. 7.** Cause of Death Ante Mortem

| Cause of Death (Ante Mortem) | Frequency | Percentage (%) |
|---|---|---|
| CHD | 2 | 11.8 |
| CVC | 1 | 11.8 |
| HMDS | 1 | 5.9 |
| NS | 5 | 29.4 |
| Prematurity | 4 | 23.5 |
| SBA | 3 | 17.6 |

SBA – Severe Birth Asphyxia, CVC – Chronic Venous Congestion, CHD – Congenital Heart Disease, HMDS – Hyaline Membrane Disease, NS – Neonatal Sepsis

**Tab. 8.** Cause of Death Post Mortem

| Cause of Death (Post Mortem) | Frequency | Percentage (%) |
|---|---|---|
| Birth trauma | 1 | 5.9 |
| CHD | 9 | 19.57 |
| HMDS | 2 | 4.35 |
| Kernicterus | 1 | 2.17 |
| NS | 18 | 39.13 |
| PNPH | 3 | 6.52 |
| Prematurity | 8 | 17.39 |
| SBA | 4 | 8.69 |

SBA – Severe Birth Asphyxia
CHD – Congenital Heart Disease
HMDS – Hyaline Membrane Disease
NS – Neonatal Sepsis
PNPH – Persistent pulmonary hypertension of the newborn

**Tab. 9.** Histological findings from routine H&E staining

| Cause of Death | Frequency | Percentage (%) |
|---|---|---|
| CVC | 30 | 65.22 |
| Fatty change | 3 | 6.52 |
| Hepatic cyst | 2 | 4.38 |
| NS | 8 | 17.39 |
| SBA | 3 | 6.52 |

SBA – Severe Birth Asphyxia
CVC – Chronic Venous Congestion
NS – Neonatal Sepsis

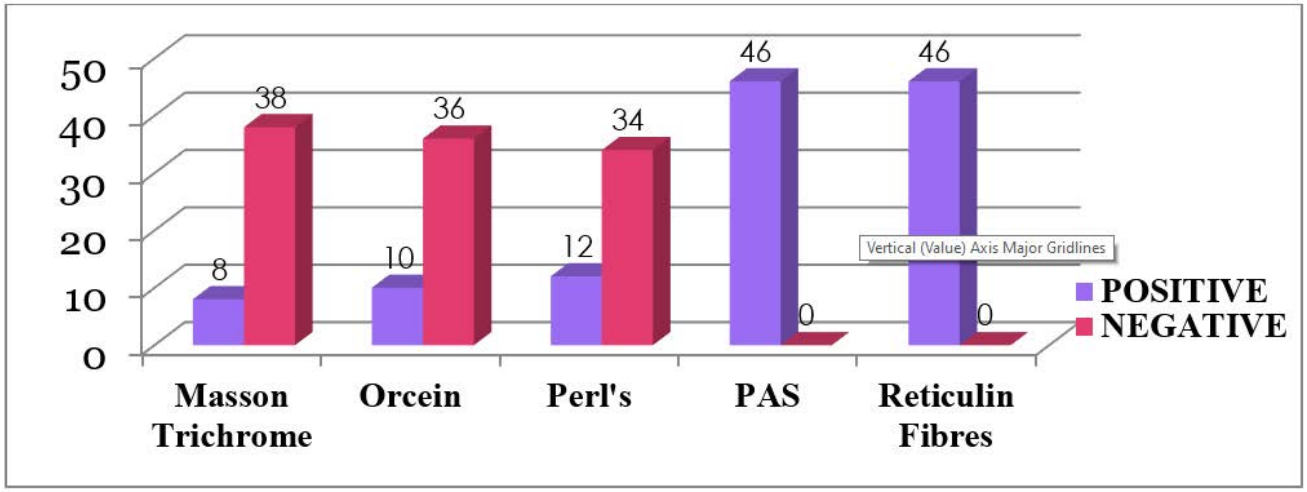


**Fig. 2.** Histochemical Stain Result

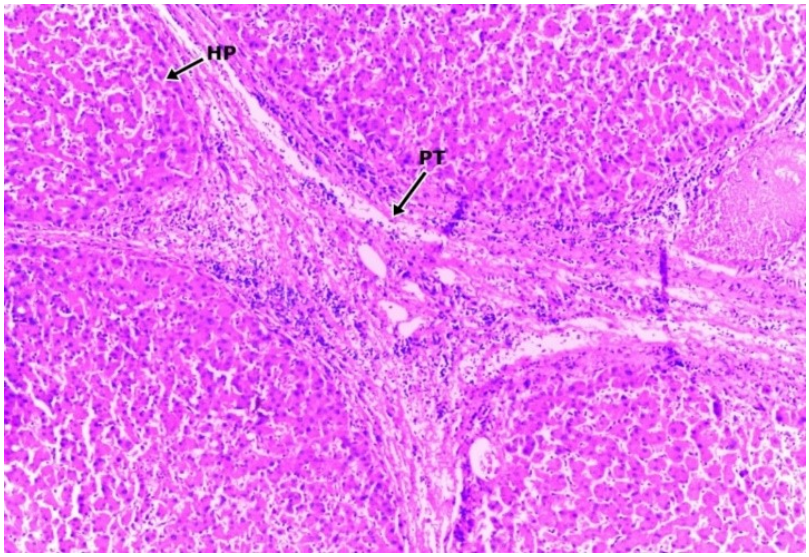


**Fig. 3.** Photomicrograph of the liver stained with haematoxylin and eosin showing an expanded portal tract area and regenerating hepatic lobules. The fibro collagenous septa are not delineated .x400

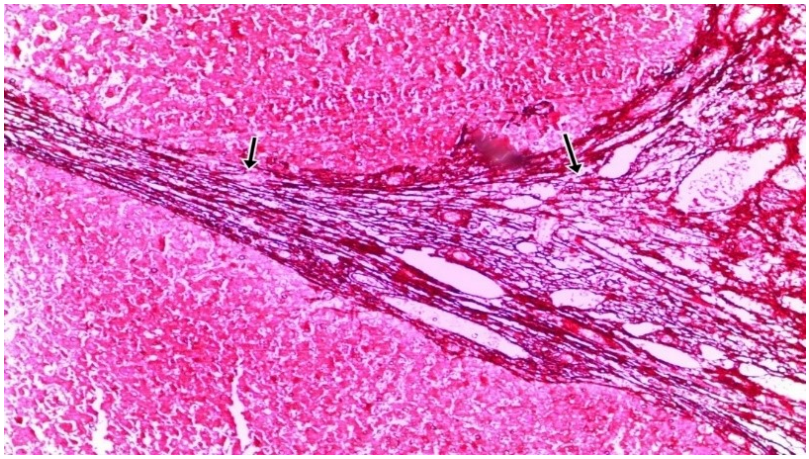

**Fig. 4.** Photomicrograph of the liver stained with Masson Trichrome showing extensive broad fibro-collagenous interconnecting bands linking the portal area to another portal area. The fibrosis is delineated shown with the stain and labelled with long arrows.x400

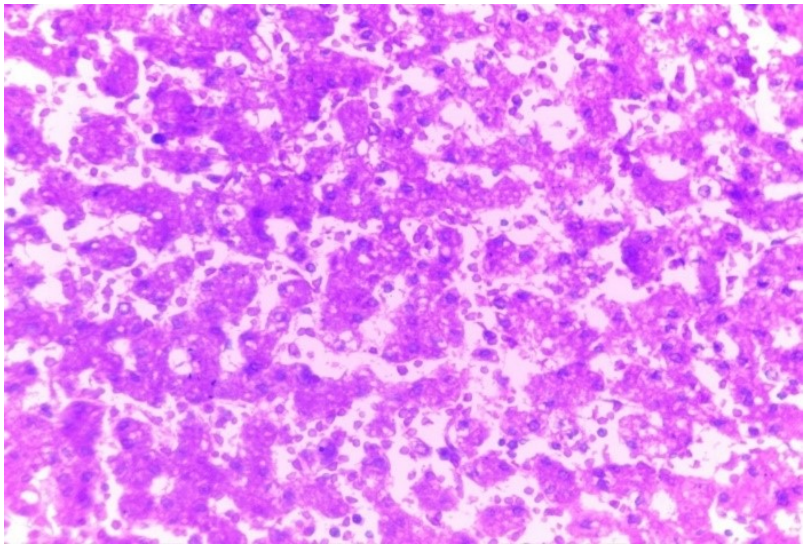

**Fig. 5.** Photomicrograph of the liver stained with Haematoxylin and eosin showing prominent hepatocytes (Long arrow) and prominent sinusoidal spaces (Shorter arrow). The stain did not indicate the presence of hepatitis virus x400

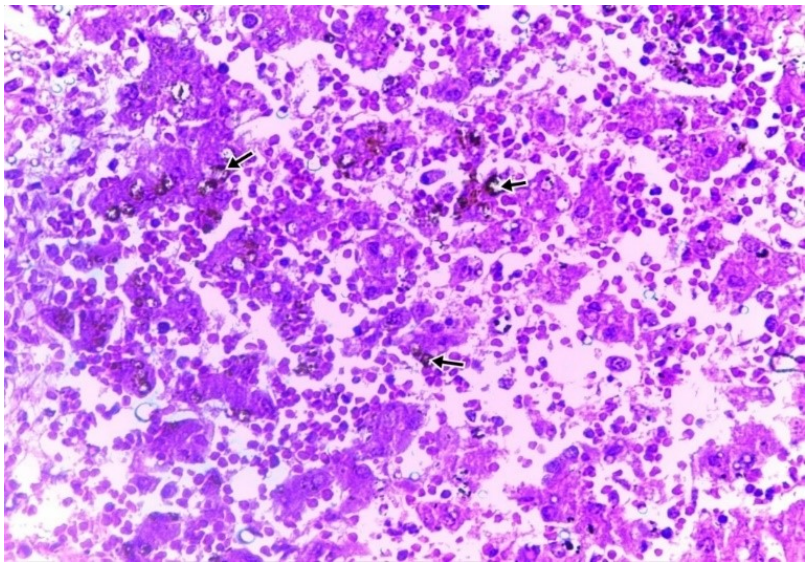

**Fig. 6.** Photomicrograph of the same liver stained with Orcein showing dark brown pigments of hepatitis B virus x400

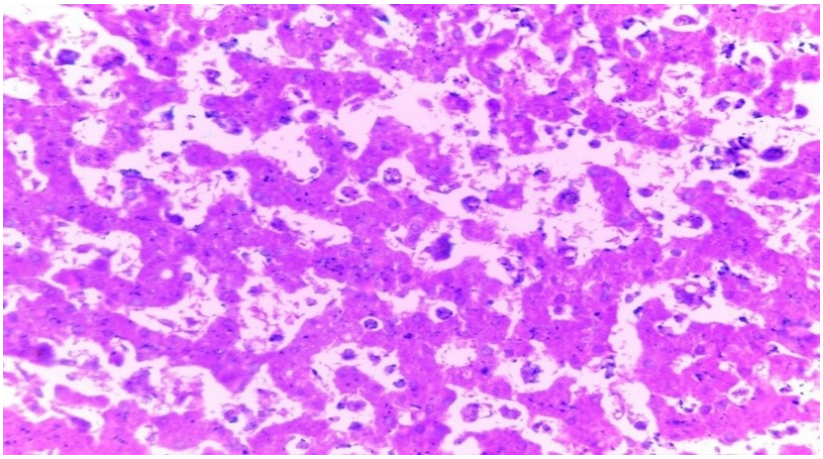

**Fig. 7.** Photomicrograph of the liver stained with Haematoxylin and eosin shows prominent hepatocytes (arrowhead) with abundant cytoplasm and basophilic nuclei. The sinusoidal spaces are dilated (Long arrow). The glycogen within the hepatocytes is not shown by H&E staining technique x400

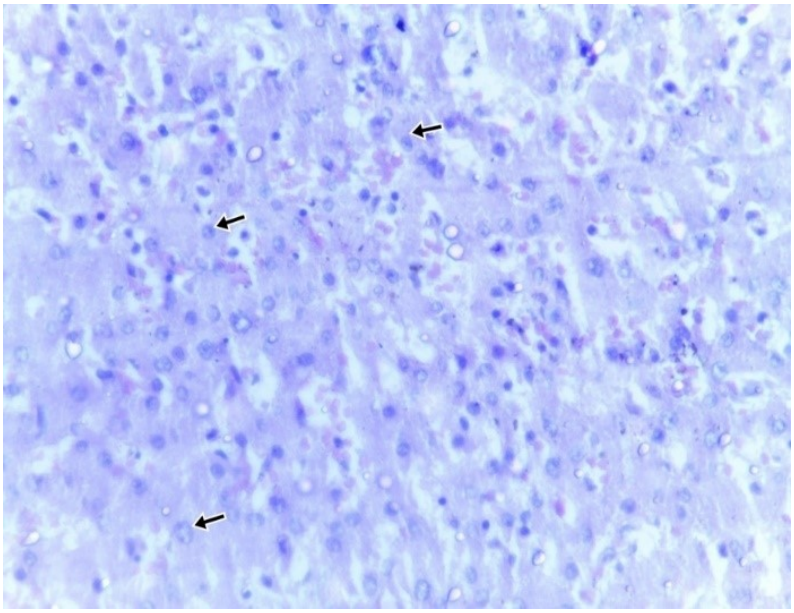

**Fig. 8.** Photomicrograph of the liver stained with PAS showing positive staining for glycogen within the cytoplasm of the hepatocytes. The cytoplasm stained faintly bright pink x400

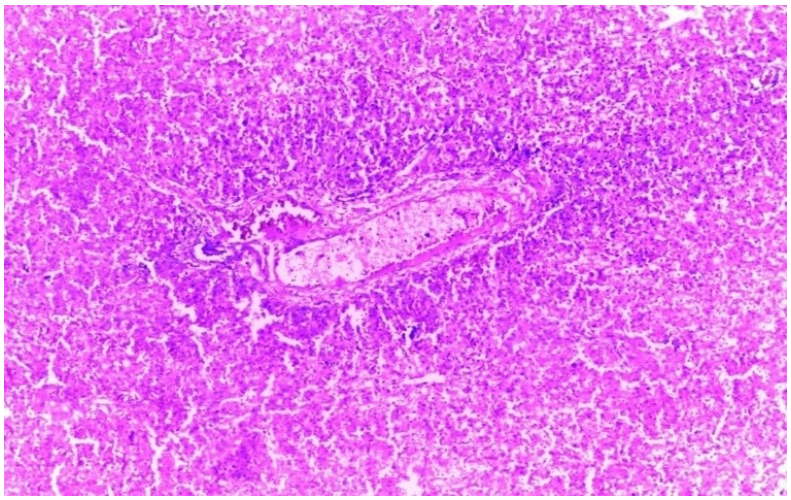

**Fig. 9.** Photomicrograph of the liver stained with haematoxylin and eosin showing prominent plates of hepatocytes (Long arrow) and portal tract (shorter arrow). The reticular connective framework is not distinct x400

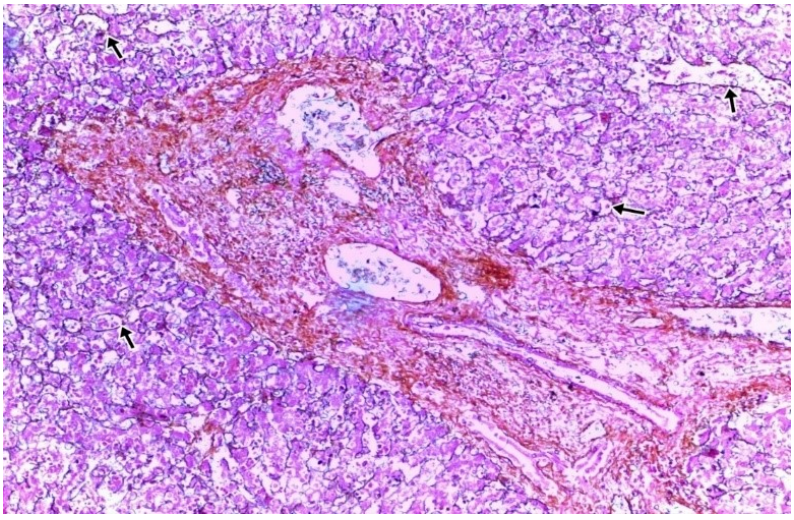

**Fig. 10.** Photomicrograph of the liver stained with reticulin showing preserved liver architecture The reticulin fibres are well outlined by the stain and wrapped around the hepatocytes as indicated by the arrows

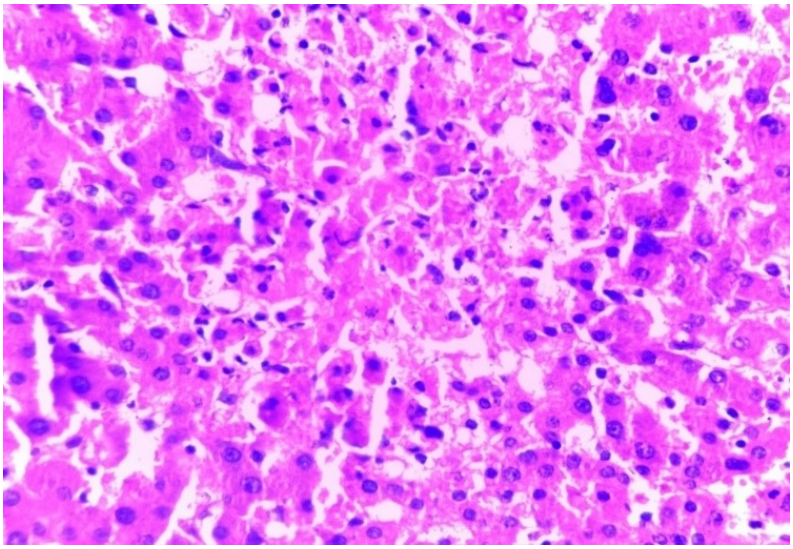

**Fig. 11.** Photomicrograph of the liver stained with haematoxylin and eosin with prominent plates of hepatocytes (long arrows). The hepatocytes have an eosinophilic cytoplasm and basophilic nuclei. Hemosiderin pigments and iron pigments are not stained by H&E staining technique x400

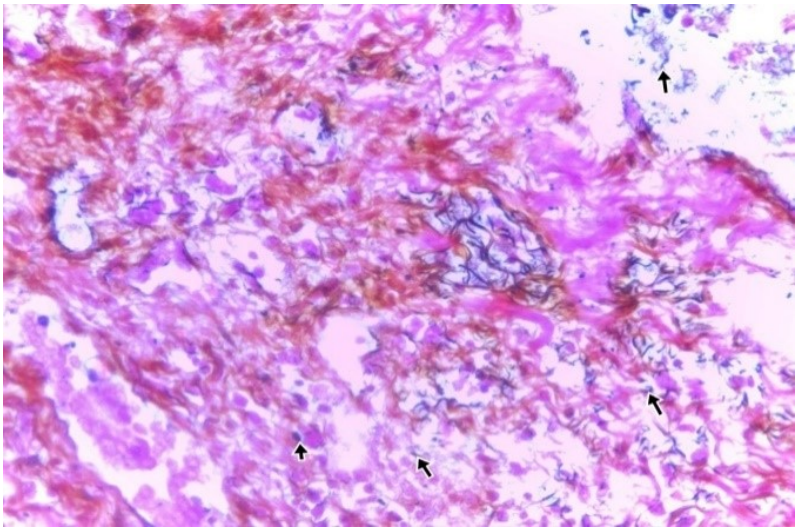

**Fig. 12.** Photomicrograph of the liver stained with Pearl's stain showing faint bluish-blue colour differentiation. This indicates the presence of Iron pigments which is not seen with H&E stained above x400

## DISCUSSION

Special staining techniques contribute significantly to the determining of the post-mortem cause of death when compared with haematoxylin and eosin staining technique [13]. The post-mortem causes of death become apparent following special stains [14]. In addition to the standard haematoxylin and eosin staining, the examination of a liver biopsy necessitates the application of additional staining techniques to highlight the architecture, degree of fibrosis, and certain other significant aspects including pigments and viral inclusions [15]. Liver biopsies are expedient in diagnosing the disease, the extent or severity of the disease and the pertinence of patients for treatment. The sternness of hepatitis and cirrhosis for example is based on the extent of inflammation, and fibrosis present in the sample [15].

In this study, haematoxylin and eosin staining consistently showed chronic venous congestion in most of the specimens collected but the exact mechanism is not fully known. The indispensability of using special staining techniques was highlighted in some samples, Perl's Prussian staining was positive in four (8.70%) of the samples indicating increased haemolysis of red blood cells that might have been a result of congenital malaria infections. This was completely missed by routine H&E examination. Perinatal mortality due to malaria is put at 1500/day in Africa and the index is relatively high in the sub-Sahara region (WHO, 2016). A study conducted at the University of Calabar Teaching Hospital by Ekanem et al (2008) on the prevalence of congenital malaria among neonates with suspected sepsis [16]. Its outcome among 546 in-born neonates reported a prevalence of 13% which indicated that congenital malarial is not uncommon among neonates in the centre. A similar study conducted at the University College Hospital; in Ibadan in South-western Nigeria reported an elevated prevalence of plasmodium falciparum parasitaemia with a prevalence reported as 23.7% [17].

Orcein staining was positive for a significant percentage (21.74%) of the samples displaying features of congenital hepatitis (HBsAg viral inclusions) which was not obvious in the previous diagnosis reported from H&E. With the advent of immunostaining for assessment of HBsAg and HBsAg, the high cost and non-availability of these markers leave low resource laboratories with no alternative but to use Orcein staining technique. Positive hepatocytes give a worthwhile clue to the possible presence of biliary diseases such as primary biliary cirrhosis and primary sclerosing cholangitis, a metabolic disorder of copper metabolism (Wilson's disease) in a non-cirrhotic liver [18].

Orcein staining of liver biopsy specimens is of additional value for the diagnosis of chronic viral hepatitis when strongly positive. Weakly positive staining reactions must be interpreted with cau-

tion and correlated with the serologic and clinical correlation with the serologic and clinical pictures. The method may also be of value in distinguishing acute from chronic hepatitis where histologic changes are minimal. Orcein staining may help determine the aetiology of cirrhosis, including those cases associated with hepatoma. Masson trichrome stain used to assess fibrosis, which gives important information about the stage and progression of the disease was found to be positive in a significant percentage (17.39%) of the samples, indicating the degree of fibrosis in the liver. A large number of liver diseases such as hepatitis B and C viral infections, fatty liver disease, alcoholic liver disease and chronic biliary diseases show the formation of fibrous tissue including bridging fibrous septa leading to the end-stage process called cirrhosis. The stain could be used to make treatment decisions; utilized to assess the effect of therapy, the treatment in these disease processes aims to halt the progression of fibrosis.

The PAS stain is based on exposing aldehyde groups in sugars by oxidation with periodic acid. The exposed aldehyde groups react with the chromophores in Schiff's reagent to produce a bright pink colour. The PAS stain demonstrates glycogen, neutral muco-substances and basement membranes. In this study, the entire samples (100%) stained positive for PAS stain. Demonstrating bile duct basement membrane damage in biliary diseases and probably the presence of alpha-1 antitrypsin globules or the presence of storage cells [11-15].

Reticulin fibres are thin fibres composed of collagen III which form a delicate stromal network in many organs [19]. The reticulin network is particularly rich in the liver and can be seen along the hepatic trabecula. Since reticulin provides stromal support for the parenchyma, the reticulin stain provides important information about the architecture of the liver [20]. The reticulin fibres that surround injured and necrotic hepatocytes collapse in the space that results. Thus, reticulin staining indicates localized hepatocyte loss in those areas. As reticulin collapses, significant patches of cell necrosis are seen [15]. In this study, the entire sample (100%) demonstrates the architecture of the liver, hepatocyte loss or necrosis and proliferation of the hepatocytes.

## CONCLUSION

The application of histochemical techniques to laboratory investigations of tissue samples can yield considerable information. In this study, the relevance of histochemical analysis cannot be overemphasised as some of the samples revealed more vital information which was not obvious by the routine H&E technique. Histochemical analysis of the liver tissue can give insight into liver function and can pinpoint and localize a wide variety of carbohydrates, proteins, lipids and enzymic activities.

There are several advantages to the use of histochemical analysis for clinical diagnosis, the result of the tests can be assessed concerning the structure of the liver as is the case in this study and the economy of use when compared to immunohistochemical analysis.

Histochemical analysis in this study helps to ascertain enzyme activities and localization, indicating neonatal livers that have normal activity and the ones with deficient activities.

Special stains are very important in the interpretation of liver biopsies. The trichrome stain imparts valuable information about the stage of chronic liver diseases and the effect of treatment. Reticulin stain is used for the assessment of hepatic microarchitecture. The PAS stain with diastase highlights the storage of abnormal metabolites and alpha-1 antitrypsin. The Perl's stains allow the detection of intracellular deposits of iron and copper respectively. Orcein stains demonstrate the presence of hepatitis B infection, copper deposition in Wilson's disease and chronic biliary diseases. Special stains should be routinely utilized for the interpretation of liver biopsy as their advantages are enormous has demonstrated by this study.

## RECOMMENDATION

The use of special stains in neonatal liver interpretation is a necessity as demonstrated by this study. Special stains should be routinely put to use in liver biopsy reporting, it is easy to use and affordable as well.

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# APPENDIX

## Principles of staining

The first step in the process of preparing a specimen for staining is fixation. Fixation, or protein stabilization, preserves the specimen in a reproducible manner that most closely resembles its living state. Next, the specimen must be dehydrated, cleared and infiltrated with a substance that will allow sectioning, or cutting, of the specimen. Sectioning, or slicing the specimen into sections typically 3 μm–5 μm, is the final step in preparing the specimen for staining. These thin sections are placed onto glass slides, dried and then stained.

For this research, previously embedded blocks were re-embedded and sections of about 3 μm-5 μm were produced for the various special staining procedures.

## Materials

Latex examination, Microscope slides, Microscope, Microtome, Hot plate, Water bath, Slide racks, Crucibles for staining, Kidney plates, weighing balance, Measuring cylinder, pH meter, Hot air oven, Forceps, Bunsen burner, Tissue cassettes, Wire gauze, Tissue paper.

## Chemicals and reagents

Embedding medium, 10% formalin, Bouin's solution, Formaldehyde (37%-40%), Glacial acetic acid

Haematoxylin, Eosin, 29% ferric chloride, Hydrochloric acid, Distilled water, 95% alcohol, Biebrich Scarlet, Acid fuchsin, 5% phosphomolybdic acid, 5% Phosphotungstic acid, Aniline blue

Alcohol, 0.5% Periodic Acid, Orcein Solution, Potassium Permanganate, Sulfuric acid, Oxalic acid, Neutral red, Aqueous potassium ferrocyanide, Potassium permanganate, Ferric ammonium sulfate, Silver Nitrate, Sodium Hydroxide, Aluminum sulfate, Alcian Blue, Schiff's reagent, Basic fuchsin, Sodium Metabisulfite.

# HISTOCHEMICAL STAINS

The special stains used in the research are;

## Masson's Trichrome Staining Protocol for Collagen Fibers

### Description:

This method is used for the detection of collagen fibres in tissues such as the skin, heart. On formalin-fixed, paraffin-embedded sections, and may be used for frozen sections as well. The collagen fibres will be stained blue and the nuclei will be stained black and the background is stained red.

### Procedure:

The sections were deparaffinized and rehydrated through 100% alcohol, 95% alcohol 70% alcohol. The sections were then washed in distilled water. The tissues were then refixed in Bouin's solution for 1 hour at $56^0$C to improve staining quality. The sections are rinsed in running tap water for 10 minutes to remove the yellow colour. The sections are then stained in Weigert's iron hematoxylin working solution for 10 minutes and rinsed in running warm tap water for 10 minutes. The sections are washed in distilled water. Staining in Biebrich scarlet-acid fuchsin solution for 15 minutes was done. After staining in Biebrich scarlet-acid fuchsin solution for 15 minutes, the sections were washed in distilled water. The sections were then differentiated in the phosphomolybdic-phosphotungstic acid solution for 15 minutes or until the collagen is not red.

The sections are transferred directly (without rinse) to an aniline blue solution and stained for 10 minutes. Then briefly rinsed in distilled water and differentiated in 1% acetic acid solution for 5 minutes, followed by washing in distilled water.

The sections are dehydrated very quickly through 95% ethyl alcohol, and absolute ethyl alcohol (these steps wiped off Biebrich scarlet-acid fuchsin staining) and the sections were then cleared in xylene. A resinous mounting medium (DPX) was used to mount the slides with coverslips. Collagen stains blue, Nuclei stains black, Muscle, cytoplasm, and keratin all stain red

# ORCEIN STAIN

## Orcein Stain - Inclusion Bodies - Hepatitis B Antigen

### Purpose:

Viruses themselves are very small and visible only with the electron microscope. Viruses can be described as parasites since they can only reproduce in host cells. These virus particles inside host cells are called "viral inclusion bodies" and are visible with light microscopy. The HBsAg (hepatitis B surface antigen) occurs most frequently as cytoplasmic inclusions in hepatocytes. Kupffer cell cytoplasm may also show the antigen.

If many hepatocytes are affected, the antigen appears as fine granules either diffusely spread throughout the cytoplasm or concentrated in the cytoplasm peripheral to the sinusoid space. This is called the 'ground glass' appearance. In single-cell involvement, the HBsAg appears as oval, round, or irregularly shaped aggregates in the cytoplasm, especially in the perinuclear region.

This procedure also stains copper-associated proteins.

### Procedure for Orcein Staining:

Orcein staining was carried out as follows:

The sections were deparaffinized and hydrated in distilled water then dipped in Potassium permanganate solution for 10 minutes. The sections are washed in water and after washing, the sections were rinsed in 5% oxalic acid, until colourless.

Afterwards, the sections were washed in tap water and then rinsed in distilled. The sections were dipped in 0.5% Periodic acid for 5 minutes and washed in tap water and then rinsed in distilled. The sections were then rinsed in 70% alcohol, dehydrated, cleared, and a coverslip was placed.

HBsAg, elastic fibres stained dark brownish-purple, Copper associated proteins stained dark purple and the background was light brownish-purple.

## Perl's Prussian Blue Staining Protocol (Perls M, 1867)

### Principle:

Dilute mineral acid hydrolysis releases ferric ions from protein-bound tissue deposits, which, in the presence of ferrocyanide ions, are precipitated as the highly coloured and highly water-insoluble complex, potassium ferric ferrocyanide, Prussian blue.

$FeCl3 + K4Fe(CN)6 = KFeFe(CN)6^{-} + 3KCl$

Ferrous ions do not produce a coloured reaction product and thus are excluded from the visualization. Tissue deposits containing ferric ions are invariably haemosiderin.

In the original method, Perl's applied the ferrocyanide and acid as separate reagents. The "mixed method" (as written here) is well suited for a routine laboratory, but it must be kept in mind that heavy deposits of haemosiderin in tissue sections may lead to leaching of the coloured end product, with subsequent artefactual background staining of collagen.

Asbestos is the name given to a special form of silica which exists in the form of long, thin, crystalline fibres. The fibres become coated with protein which contains haemosiderin and therefore appear brown on unstained and H&E sections and blue by Perl's Prussian blue reaction. The asbestos fibres with their protein are known as "asbestos bodies" and the characteristic birefringence is lost.

## Procedure for Perl's Prussian blue staining

The sections were hydrated with distilled water. On a rack, equal parts mixture of ferrocyanide and hydrochloric acid was flooded on the sections for 10 minutes. The slides are washed in several changes of distilled water for 5 min. A filtered neutral red stain was used for counterstain for 1 min. The sections were then rinsed in distilled water. Afterwards, the sections were rapidly dehydrated in absolute alcohol, cleared and mounted.

## RESULTS

Ferric salts stain deep blue, Nuclei stained red, Erythrocytes stained yellow and asbestos bodies stained blue/black.

## Reticular fibres (Gordon and Sweet 1936)

### Purpose:

A silver impregnation technique that demonstrates reticular fibres. Reticulum is a support function of the body and is abundant in the liver, spleen, and kidney. In a normal liver, the fibres are well-defined strands, but necrotic and cirrhotic livers show discontinuous patterns. Reticulum also forms characteristic patterns in relationship to certain tumour cells.

### Principle:

The tissue is oxidized, and then sensitized with the iron alum, which is replaced with silver. The silver is reduced with formalin to its visible metallic state.

## The procedure used for Reticular Fibers (Gordon and Sweet's Method)

The slides were deparaffinized and hydrated with distilled water. The sections are then dipped in Potassium permanganate solution for 5 minutes. Washed in water and rinsed in 5% oxalic acid until clear and re-washed in distilled water. The sections are rinsed in Iron alum solution for 10 minutes and then rinsed in running tap water and rinse in 3 changes of distilled water. In Silver solution, 7 dips, excess solution was shaken off the slides and washed in two changes of distilled water in three quick dips and then dipped in 10% formaldehyde solution until grey-black for 30 seconds and washed in distilled water. Followed by 0.5% Gold chloride for 1 minute and rinsed in distilled water. The sections were then dipped in 5% hypo for 1 minute and washed in tap water followed by nuclear-fast red solution for 5 minutes and washed in running tap water. Then dehydrated, cleared, and coverslipped.

### Expected Results:

Reticular fibres stains black and nuclei stained red

## PAS - Mcmannus' Periodic Acid Schiff's – Glycogen

### Purpose:

Glycogen is present in the skin, liver, parathyroid glands and skeletal and cardiac muscle. The PAS stain is used for the demonstration of basement membranes, fungus-secreting adenocarcinoma from undifferentiated squamous cell carcinoma, and mucosubstances secreted from the epithelia of various organs. A routine stain for liver and kidney biopsies.

### Principle:

The PAS stain is a histochemical reaction in that the periodic acid oxidizes the carbon-to-carbon bond forming aldehydes which react to the fuchsin-sulfurous acid which forms the magenta colour.

## The procedure used for Periodic Acid Schiff's Staining

The sections were deparaffinized and hydrated to distilled water and dipped in 0.5% Periodic acid for 5 minutes and rinsed in distilled water. The sections were stained with Schiff's reagent for 30 minutes and washed in running tap water for 5 minutes. The sections were counterstained in hematoxylin for 3 minutes and rinsed in distilled water. The sections were dehydrated in alcohol, cleared, and cover-slipped.

### Expected results:

Glycogen stains magenta and nuclei stained blue.