ADDIS ABABA UNIVERSITY

COLLEGE OF HEALTH SCIENCE

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DEPARTMENT OF PATHOLOGY

# INVESTIGATING THE ROLE OF LIQUID DISH WASHING SOAP AS A SUBSTITUTE FOR XYLENE IN ROUTINE HEMATOXYLIN AND EOSIN STAINING: COMPARATIVE STUDY

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This is to certify that the thesis prepared by Dollar Nureddin, entitled: **Investigating the role of liquid dish washing soap as a substitute for xylene in routine Hematoxylin and Eosin staining procedure for biopsy sections: comparative study** and submitted in fulfillment of the requirements for the Degree in Master of Science (Histotechnology) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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**Table of Contents**

## List of tables

## List of Figures

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## List of abbreviations/acronyms

AAU - Addis Ababa University

ACGIH - American conference of Governmental industrial hygienist

BEI - Biological exposure index

NCCLS - The national committee for clinical laboratory standard

DRERC - Departmental Research Ethics Review Committee

$dH_2O$ - Distilled water

DWS - Dish washing Soap

ECAE - Ethiopian Conformity Assessment Enterprise

FFPE- Formalin Fixed Paraffin Embedded

H & E - Hematoxylin and Eosin

MHA - Methyl hippuric acid

OSHA- The Occupational Safety and Health Administration

PAS- Periodic acid Schiff

PEL - Permitted exposure limits

PI - Principal Investigator

SPSS - Statistical Package for Social Sciences

TASH – Tikur Anbessa Specialized Hospital

TWA - Total Weighted Average

VIP - Vacuum Infiltration Processing

XAF - Xylene Alcohol Free

XMF- Xylene Methanol Free

## Operational Definition

- Nuclear staining: the intensity of basophilia in a cell.
- Cytoplasmic staining: the intensity of eosinophilia in a cell.
- Clarity of staining: devoid of cloudiness throughout the section.
- Uniformity of staining: devoid of patchy staining and out-of-focus areas throughout the section.
- Crispiness of staining: being able to see well delineated nuclear membranes and sharply stained condensed chromatin against an unstained nucleolus.
- Diagnosable section is a section that has been reported as adequate for three or more of the above parameters by the pathologists.

## Summary

**Background:** One of the most important chemical in histopathology laboratory but potentially hazardous to the technologists is xylene. Histotechnologists and other workers in the histology laboratory are exposed to xylene during various procedures, especially during deparaffinizing tissue sections. Lack of proper disposal system of xylene and the laboratory workers poor preventive mechanism towards the chemical in daily activities are some of the reasons that make the impact of the exposure worse.

**Objective:** To investigate the role of liquid dish washing soap as a substitute for xylene in routine Hematoxylin and Eosin (H&E) staining procedure for biopsy sections.

**Materials and Methods:** One hundred forty one tissue blocks were used. Two sections from each paraffin block, a total of two hundred eighty two sections with 4µm thickness were included. From each pair of section, one was stained with conventional (routine) H&E staining procedure and the other with xylene-alcohol free (XAF) H&E staining procedure. Slides were scored using five parameters: Nuclear, cytoplasmic, clarity, uniformity, and crispness of staining. Slides that fulfilled at least three of the aforementioned parameters were considered adequate for diagnosis. Three different pathologists evaluated the slides independently. Z test and McNemar's test were used to compare the difference between the two methods, $p$-value < 0.05 considered significant.

**Result:** From the three, one pathologist reported that there is no difference between the paired sections on nuclear staining ($Z$=0.5, $p$>0.05). As reported by pathologists II($Z$ =0.6& 0, $p$ >0.05) and III($Z$ =0.9 & 0.3, $p$ >0.05), there is no significant difference between the paired sections on cytoplasmic and uniformity of staining. Pathologist III reported all slides stained by both methods as "clear", indicated no difference between conventional and XAF H&E stained slides. Pathologist I($Z$ =1.7, $p$ >0.05) and III($Z$ =0, $p$ >0.05) noted that both staining methods showed a crisp staining and there is no significant difference. Finally pathologists II($Z$ =1, $p$>0.05) and III ($Z$ =0.6, $p$ >0.05)showed that there is no difference between the two methods stained sections for diagnosis. All the above results are shown by "$Z$" test because both "$Z$" test's and McNemar's test $p$ value lead to similar statistical conclusion. However, the result of pathologist I for

crispiness of staining showed discrepancy, while "*Z*" test showed no significant difference, McNemar's test indicated agreement between the methods.

**Conclusion:** Removing xylene from the routine staining procedure has many advantages. Replacing it with safer, cheaper and easily disposable reagent is ideal. Therefore, considering the advantages of the new method with regard to the pathologists report in this study, it is possible to say that XAF H&E staining procedure has the potentiality of being the new routine procedure in histology laboratory.

**Key words:** Xylene, Deparaffinizing, Histotechnology, H & E, McNemar's test, Z test

# 1. INTRODUCTION

## 1.1. Background

Currently many complex and advanced techniques are being introduced in pathological practice to make more accurate and consistent diagnosis. There are still old methods that are abundantly used for routine diagnostic histology work; Hematoxylin and Eosin (H & E) is one of them [1].

H&E is a primary contrast method in medical diagnosis of biopsy specimens and is still considered essential for recognizing tissue types and morphological indicators of diagnostic cancer pathology. It is remarkably effective and works well with a variety of fixatives and is used to discriminate between a broad range of cytoplasmic, nuclear and extracellular matrix features [1,2,3].

Xylene and graded alcohol are major components in the H&E staining procedure, which used to carry out the intermediate steps of deparaffinization, rehydration and dehydration of tissue sections during the procedure [2,3]. In staining procedures, xylene's excellent dewaxing and clearing capabilities contribute to brilliant stained slides. Other than staining, it is used in histological laboratories for tissue processing and cover slip mounting [2,4].

Xylene is a colorless, sweet smelling liquid or gas occurring naturally in petroleum, coal, and wood tar. It is widely used in industries and medical technology as a solvent [4]. Total or mixed xylene that is used in histology laboratory generally contains about 40–65% m-xylene and up to 20% each of o-xylene, p-xylene, and ethylbenzene [5].

Xylene is extremely hazardous. Exposure to xylene can occur via inhalation, ingestion, eyes and skin contact. Reports among subjects chronically exposed to xylene vapors including histology technicians showed: respiratory tract effects (significant irritation of nose and throat), gastric discomfort (nausea and vomiting) cardiovascular effects (flushing, palpitations and chest pains) and neurological symptoms (anxiety, dizziness, inability to concentrate and forgetfulness) [6,7].

Xylene inhaled by a woman can reach a developing fetus and can contaminate her breast milk [5]. It has been reported that there were cases of spontaneous abortions in female pathology technicians who were exposed to formalin and xylene [8]. Frequent or prolonged skin contact

with xylene can cause irritation, dermatitis, dryness, flaking and cracking of the skin. Damaged skin allows greater absorption of chemicals [9,10]. Laboratory workers exposed to xylene for 1.5 to 18 years were described as having the equivalent of general poisoning disorders [11].

Besides occupational exposure, the principal pathway of human contact is via soil contamination. Xylene can leak into the soil, surface water, or ground water, where it may remain for months before it breaks down into other chemicals [5,12]. Xylene with other organic solvents in the environment is a silent threat to human health. Studies that included animal subjects mentioned that xylene exposure could cause kidney failure, liver defects, cancer, nervous disorders, birth anomalies, circulatory problems, and sterility [13].

In a histology laboratory where there is an exposure of xylene, the preventive methods could be substitution, usage of proper personal protective equipment or installing local exhaust ventilation. Monitoring xylene by measuring xylene vapour in the laboratory and evaluating the content of the major metabolite of xylene (methyl hippuric acid-MHA) in the urine samples of exposed individuals is very important [14].The Occupational Safety and Health Administration (OSHA) of the United States department of labor permissible exposure limits (PEL) for xylene is 100 ppm as an 8 hour time-weighted average concentration. And the American conference of governmental industrial hygienist (ACGIH) recommend biological exposure index (BEI) limit of MHA to be 20mg/dL in the urine [4,15].

After the hazardous effects of xylene became indisputable in the 1970s, many potential substitutes became available in order to make a xylene-free environment in laboratories, such as limonene reagents, aliphatic hydrocarbons, aromatic hydrocarbons, olive oil, vegetable oil, and mineral oil. But the substitutes are either very expensive or more hazardous than or as hazardous as xylene and some of them are less effective in their chemical roles. Later, liquid dish washing soap got attention by many researchers of the field [11,14,16].

Liquid dish washing soap mainly consists of a surfactant or a mixture of surfactants with cleaning properties in dilute solutions. The surfactants compound includes at least one group having an affinity for markedly polar surfaces, ensuring in most cases solubility in water and another group which has little or no affinity for water. Liquid detergent for hand dish washing

soap has a neutral pH and it does not cause any irritant or undesirable effect on the skin and hands [17].

Different research works showed that the dewaxing performance of hot liquid DWS and xylene were at the same level [1,11,18-24]. Falkeholm *et al*., is the first team to experiment with these in 2001.

Thus, the present study is designed to perform H&E staining procedure by using the easily available liquid DWS as a deparaffinizing agent and compare the quality with that of the conventional H&E stained slides which contain xylene and ethanol in the procedure.

## 1.2. Statement of the problem

Pathology laboratory technicians are routinely exposed to xylene during procedures like tissue processing, staining, cover slip mounting and cleaning tissue processing machines [4,14].

Developed countries like United States (US) have provisions like OSHA and ACGIH, these bodies have established standards for biological exposure limits, for monitoring exposure and managing the disposal and recycling of xylene. Therefore, there are continuous monitoring of histology laboratory workers for their exposure limit to xylene in developed countries [11,19].

Additionally, the knowledge of xylene toxicity urged histology laboratories to practice the preventive methods in developed countries. For example, in 2007, 41% of United States (US) histology laboratories use xylene substitutes for tissue processing and 79% and 62% of them use machines for staining and cover slip mounting respectively [11].

However, the pathology laboratories in most developing countries, including India have neither any mechanism for monitoring the exposure nor any standardized methods for disposal of xylene like the developed countries [19].

In 2015, in Ethiopia, many government histology laboratories were staining and cover slip mounting manually [25]. Even though monitoring the permitted exposure limit (PEL) and the biological exposure index (BEI) of the laboratory workers is crucial for the workers, there is no monitoring system in our laboratory. In addition, there is poor preventive mechanism due to shortage of local exhaust ventilation with proper hood and unavailability of personal protective equipments. There is no standard disposal system for hazardous chemicals in pathology laboratory, indicating the potential environmental pollution that could be a threat for the general population at large. In addition to its toxicity xylene is expensive for countries like Ethiopia, which import it from abroad.

Therefore, there is no better way than replacing the expensive, toxic and hazardous xylene with safer, cheaper and eco-friendly substitute. This research aims to assess if dish washing soap (DWS) is a good substitute for xylene in routine staining procedure to make our laboratory a safer place to work and create less polluted environment in general.

### 1.3. Significance of the study

In Ethiopian histopathology laboratories, there are no established standards for biological exposure limits, for monitoring exposure and managing the disposal and recycling of xylene. Hence, besides the laboratory workers due to massive environmental pollution the hospital workers and the general population are also at risk. So finding a safe xylene substitute is an ideal option. This research presented liquid dish washing soap as a competent and safe option to replace xylene in the H&E staining methods.

## 2. LITERATURE REVIEW

A research team, Falkeholm L. *et al.,* conducted a research in Sweden in 2001 whether or not the xylene-free sections are equivalent to conventionally processed sections. From breast, intestine and skin biopsy, 30 paraffin blocks were used. From each block three sections were prepared for three stain type. Each slide were examined and scored by nine pathologists from three hospitals. Wilcoxon signed rank and Cohen's kappa agreement test were the statistical tools used. Xylene free sections ranked as good as or better than their conventional counterparts in 74% of the comparisons. H&E and PAS sections were equivalent while Van Gieson's stained sections tended to be downgraded for the xylene-free slides. Specifically for H&E slides, from 270 matched pairs, the conventional method agreed with the XAF in 211 pairs [1].

Annals of diagnostic pathology published a paper entitled "Histology without xylene" by Buesa RJ *et al.,* in 2009. They experimented, compared and discussed many xylene substitutes versus xylene for tissue processing, staining and cleaning purposes. For staining, 33 pair of sections were evaluated by seven participants and F test of variance was used to analyze the bias between them. Finally, the result showed 1.7% dishwasher soap as a dewaxing reagent was diagnostically equivalent with xylene [11].

An experimental cross sectional study was conducted by Ankle M.R. *et al*., in 2011, in India. They used 60 paraffin blocks of routinely processed tissue specimens and prepared paired slides from each block to stain with the conventional (routine) and XMF H & E staining method. They used five parameters to score slides: Nuclear, cytoplasmic, clarity, uniformity, and crispness of staining. A single oral pathologist evaluated the slides. "*Z*" test was used to compare the proportion difference between the two staining methods. Though statistically significant difference was noted on cytoplasm, uniformity and crispiness of staining, they concluded that the XAF method produces diagnostically the same quality of slides as the conventional [19].

A study was conducted in India by Ramulu S. *et al.,* in 2012 with the same objective to the previous researches. The difference is that in this research, they used less number of blocks (50 blocks), and there was no statistically significant difference between the two methods stained slides [20].

Another similar research was done in India by Negi A. *et al.,* in 2013.They used 30 formalin-fixed paraffin-embedded (FFPE) tissues. Three different oral pathologists scored the slides and Wilcoxon matched-pairs signed rank test was used to calculate the test of significance. Though the results were found to be statistically insignificant, the mean values of the two observers for clarity, uniformity, crispness and diagnosis showed higher proportion for XAF method as compared to routine H&E method. The mean value of one observer for nuclear and cytoplasmic staining showed higher proportion for XAF method [21].

Another similar prospective study was conducted in India by Pandey P. *et al.,* in 2014. They used 100 paraffin embedded tissue blocks from different tissues and one pathologist evaluated the slides. XAF stained sections scored better for cytoplasmic (90%) and crisp staining (95%) with a statistically significant difference from the conventionally H&E stained sections. Whereas for uniformity of staining, conventionally stained sections (88%) scored over XAF stained sections (72%) ($Z = 2.82$, $P< 0.05$). For nuclear and clarity of staining, the XAF stained sections were in favor, but the difference was not statistically significant. Conventionally stained sections scored 84% for adequately of diagnosis while XAF stained sections scored 86% [18].

Another research entitled “Efficacy of 1.5% Dish Washing Solution and 95% Lemon Water in Substituting Perilous Xylene as a Deparaffinizing Agent for Routine H & E Staining Procedure” was published by Ananthaneni A. *et al.,* in 2014. Using twenty paraffin embedded tissue blocks they performed three different procedures and compare them. One section was stained by conventional H&E method and the other two sections by xylene-free H&E method. As the previous studies, the same parameters were used to evaluate the slides and one oral pathologist evaluated them. After they analyze the data using Chi-square, Kruskal Wallis ANOVA, and Mann-Whitney U test, they concluded that 1.5% dish washing solution or 95% diluted lemon water can be a substitute for xylene as deparaffinizing agent in hematoxylin and eosin procedure[22].

In 2015, similar paper was published by Sadashiv R. *et al.,* in India. Unlike the above researches, this research used 2% dishwashing soap (DWS) solution as a deparaffinizing agent. All the 50 paraffin blocks were different tissues from dead fetus. In this study except for cytoplasmic and clarity of staining, in other parameters conventionally stained slides scored better. Still, no

statistically significant difference was seen between the two methods, which made the conclusion similar to the above researches [23].

A similar study was conducted by Gisuthan B. *et al.,* in 2017. In this study, the temperature of the diluted DWS was $75^0$c unlike the other studies which were $90^0$c. Forty paraffin blocks with well preserved tissues were selected; tissue sections with necrotic material, under fixed or those showed artifacts were excluded. McNemar's test was the statistical tool used. For all the five parameters no significant difference was reported, but based on the total scores obtained for each slides the observed p value for diagnosis was 0.041, indicated a significant difference between modified and conventional method. However, they concluded like the other studies that the XAF H&E stained sections are the same with the conventional H&E stained sections [24].

As shown above, though there were major and minor differences encountered in their results, all the studies concluded that liquid DWS is a safer and more efficient alternative to xylene for routine H&E staining procedure. Some of them tried to include different kinds of tissues however; most of them had limited sample sizes. Except for very few, in most of the above researches, only one pathologist evaluated the slides. Therefore, this study of ours was intended to determine the difference between XAF and conventionally stained H&E slides using more than 100 blocks that were processed and stained in TASH, Pathology Department, with our materials and reagents using Ethiopian made liquid dish washing soap called Master®. Finally, the slides were evaluated by three experienced pathologists.

# 3. OBJECTIVES

## 3.1. General objective

The global objective of this study was to investigate the role of DWS as a substitute for xylene in routine H&E staining procedure in histology laboratory.

## 3.2. Specific objectives

To determine the difference between xylene and alcohol free H&E stained slides with that of the conventional H&E stained slides on "adequacy of nuclear staining"

To determine the difference between xylene and alcohol free H&E stained slides with that of the conventional H&E stained slides on "adequacy of cytoplasmic staining"

To identify the presence of "clarity of staining" and compare xylene and alcohol free H&E stained slides with that of the conventional H&E stained slides

To elucidate the presence of "uniformity of staining" and its difference between xylene and alcohol free H&E stained slides with that of the conventional H&E stained slides

To investigate the presence of "crispness of staining" and compare between xylene and alcohol free H&E stained slides with that of the conventional H&E stained slides

# 4. MATERIALS AND METHOD

## 4.1. Study area

This study was conducted at Tikur Anbessa Specialized Hospital (TASH), pathology department. TASH is located in Addis Ababa at Lideta sub-city opposite to Immigration office Ethiopia. It is the teaching hospital of the Addis Ababa University and the largest referral hospital in the country. TASH offers diagnosis and treatment for approximately 370,000- 400,000 patients a year.

The department of Pathology in TASH is one of the biggest histopathology centers in the country, with 7000-9000 biopsy specimen intake capacity annually. There are eight pathologists and eight laboratory workers in the department.

## 4.2. Study design

In this research, a comparative cross-sectional study was conducted.

## 4.3. Study period

The study was conducted from October 2016 to January 2018. Actual task on sample preparation, processing, staining, slide distribution and result collection took place from July 2017 to September 2017.

## 4.4. Specimen/sample

### 4.4.1. Source sample

The source sample was all biopsies that came to TASH, received by pathology department; cut sectioned, and sent in cassettes to histopathology laboratory to be processed within the study period, July 2017.

### 4.4.2. Study sample

The study sample was 141 cassettes of tissue samples that had been cut sectioned and sent to histopathology laboratory to be processed the following day.

## 4.5. Sample size and sampling method

The national committee for clinical laboratory standard (NCCLS), 2011 guideline recommends a minimum of 40 samples for method comparison. In addition to this, Westgard recommends large

sample size (100 to 200) to assess whether new method is similar to that of the comparative method [26].

According to the above recommendation, one hundred forty one biopsy specimens were selected by convenient sampling method. The VIP processing machine that was used to process the specimens could process 150 cassettes at once, but the type of cassettes that were used for this research were relatively thicker than the usual and it was impossible to insert the 9 cassettes in the cassette holding chamber of the processing machine. Due to these reason, only 141 cassettes were able to be processed at once.

### 4.6. Eligibility

#### 4.6.1. Inclusion criteria

Among the biopsies that were cut-sectioned and came to the laboratory in cassettes to be processed before the day of the processing (7/29/2017), the first 141 biopsy cassettes were used.

#### 4.6.2. Exclusion criteria

- After the cassette holding chamber of the processing machine were filled, the other biopsy cassettes were excluded.

### 4.7. Variables

#### 4.7.1. Dependent variable

- Nuclear staining
- Cytoplasmic staining
- Clarity of staining
- Uniformity of staining
- Crispness of staining

#### 4.7.2. Independent variable

- Xylene and alcohol free H & E staining method
- Conventional H & E staining method

### 4.8. Study Procedure and Data Collection

#### 4.8.1. Reagents

The xylene alcohol free H&E staining procedure used 2% Master® hand dish washing liquid detergent(DWS) for deparaffinization of biopsy slides as an alternate substitute for xylene. DWS (30 ml) was diluted in 1500 ml distilled water ($dH_2O$). Since water based reagent was used for deparaffinzation, alcohol was no longer needed in this procedure.

Mayer's hematoxylin was used for both XAF and conventional H&E staining methods. It was prepared by completely dissolving the hematoxylin, potassium alum, and sodium iodate in $dH_2O$ by warming and stirring. Then, chloral hydrate and citric acid were added, and the mixture was boiled for 5 minutes, cooled and filtered.

As a cytoplasmic stain, 1% eosin Y was used. It was prepared as 1.0% solution, with the addition of 0.5 ml acetic acid per liter.

#### 4.8.2. Tissue sample and processing

Different kinds of tissue samples were used in this research, including: bone marrow, lymph node, breast, female genital tract, gastro intestinal tract, bladder, brain, pancreas, conjunctiva, skin and lung. Within these samples inflammatory, neoplasm (both benign and malignant) and pregnancy related disorders were present. The specimens were fixed (in 10% formaldehyde) then cut sectioned to fit in a processing cassette. A total of 141 tissue cassettes were processed with conventional (routine) method (as shown in Table 1) by “Tissue Tek II” VIP processing machine. Then all 141 of them were embedded in a paraffin wax embedding media.

**Table 1:** Tissue Processing Schedule

| No | Reagents | Time | Temperature |
|---|---|---|---|
| **1** | 4% Formalin | 1 hour | $35^0$c |
| **2** | Water | 5 second | $35^0$c |
| **3** | 50% Ethanol alcohol | 1 hour | $35^0$c |
| **4** | 70% Ethanol alcohol | 1 hour | $35^0$c |
| **5** | 80% Ethanol alcohol | 1 hour | $35^0$c |
| **6** | 90% Ethanol alcohol | 1 hour | $35^0$c |
| **7** | 100% Ethanol alcohol | 1 hour | $35^0$c |
| **8** | 100% Ethanol alcohol | 1 hour | $35^0$c |
| **9** | Xylene | 1 hour | $35^0$c |
| **10** | Xylene | 1 hour | $35^0$c |
| **11** | Paraffin wax | 45 minutes | $68^0$c |
| **12** | Paraffin wax | 45 minutes | $68^0$c |
| **13** | Paraffin wax | 45 minutes | $68^0$c |
| **14** | Paraffin wax | 45 minutes | $68^0$c |

### 4.8.3. Histological procedure

From each 141 tissue paraffin blocks, two slides were prepared in order to run every specimen in both XAF and conventional H&E staining method. All 282 slides were sectioned by one person, with one microtome, and with same thickness of 4 μm. The specimen's laboratory serial number was not used rather there was a new code given, 001-141, also each number had A&B label on it. But after that the paired slides were randomly separated into two categories, while the principal investigator (PI) register the exact label of the slide to a checklist for later cross checking of slide with the staining procedure performed. Then half pair (141 slides) was stained by the conventional and the other by XAF method.

Staining of the conventional sections were preceded by rehydration, followed by dehydration in alcohol and clearing with xylene before mounting, whereas these steps were not necessary for

the XAF sections. For XAF sections staining followed immediately after deparaffinization was done using diluted 2% liquid DWS at 90°C. Finally there was Oven drying the XAF sections at 60°C prior to cover slipping.

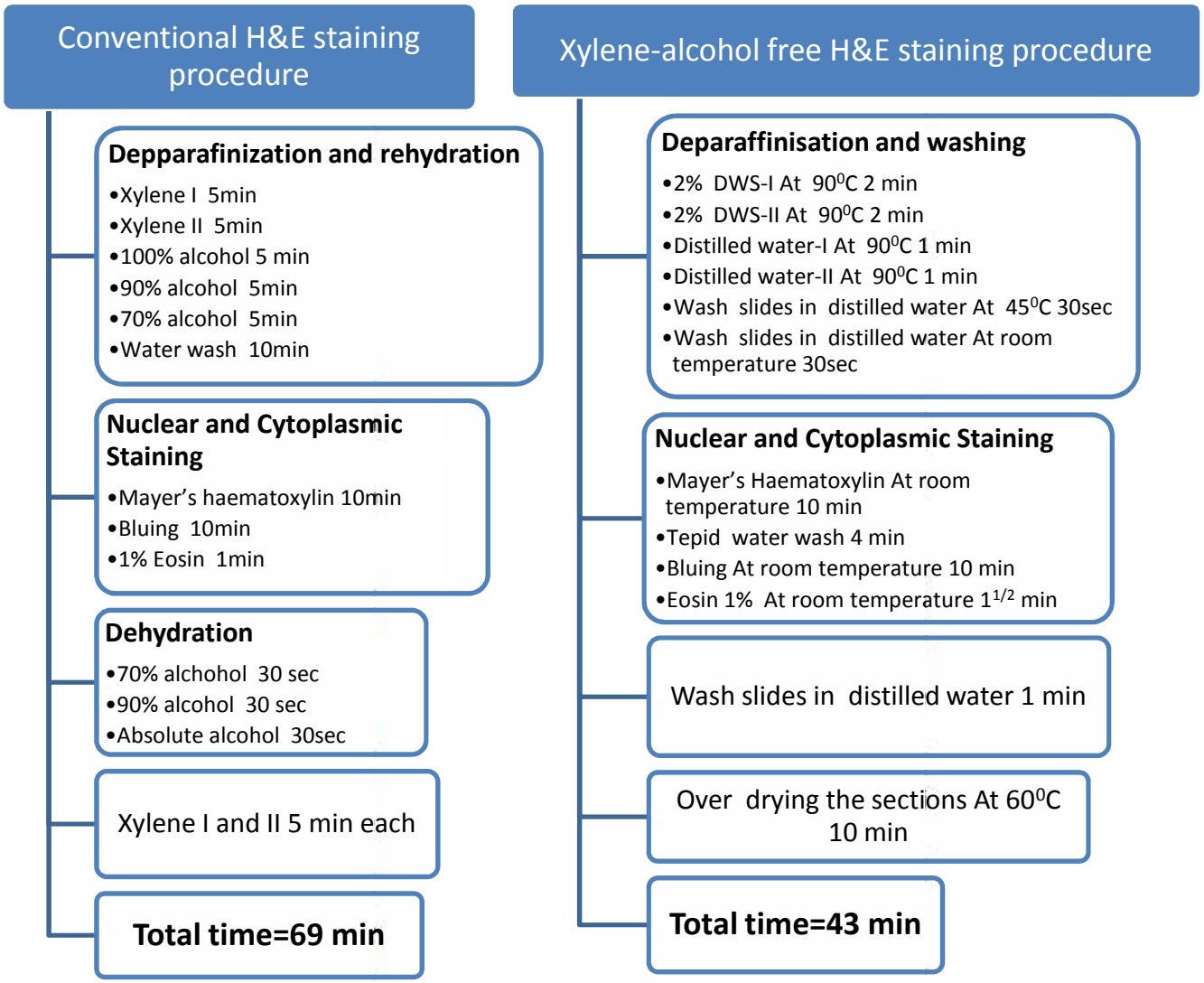


**Figure 1**: Conventional and xylene-alcohol free H&E staining procedure

### 4.8.4. Assessment of stained sections

All the 141 matched pairs were analyzed by three different pathologists referred as pathologists I, II and III. The pathologists used a checklist that contains a scoring system that has been used by other similar studies in the past [18-24]. All the pathologists had no prior knowledge of staining method employed, so the study was blinded to prevent the observer bias.

H&E stained sections were graded based on the following five parameters:

- Nuclear staining (adequate = score 1, inadequate = score 0)
- Cytoplasmic staining (adequate = score 1, inadequate = score 0)
- Clarity of staining (present = score 1, absent = score 0)
- Uniformity of staining (present = score 1, absent = score 0)
- Crispness of staining (present = score 1, absent = score 0).

A score of 0/1 were given to each of these parameters and the slides were graded by adding up the scores. Slides with a score, ≤2 were graded as inadequate for diagnosis, and the slides with score, 3-5 were assigned as adequate for diagnosis. "*Z*" test and McNemar's test were used to compare the two staining methods, $p < 0.05$ considered as significant.

### 4.8.5. Pilot studies

Before the preparation of the actual research slides, six pilot studies were performed for the sake of a better result. In each pilot 10 and more pair of slides from different type of tissue were used. To deparafinize the slides with DWS at $90^0$c different materials like, dry oven, analog and digital water baths were used. Diluted 1.7% and 2% solutions were prepared using sine® and master® dish washing soaps. From ECAE cleared detergents that were shown in their website sine® DWS was randomly selected first. Later to check the consistency of the result, another ECAE cleared DWS were used and the result was the same. Master® hand dish washing soap was used for the final work because it has less foam than sine® which makes it easy to work with. From these six pilot studies experience it was clear that;

The temperature of the DWS had to be maintained at $90^0$c, so digital water baths were used.

The concentration of the DWS is important, so 2% was used.

The depparafinization and washing time in XAF procedure had to be optimized. Therefore, the research slides were prepared based on the procedure as shown in figure 1.

Finally based on the pathologist's evaluation, the seventh pilot was done to understand and try to improve the staining quality.

### 4.9. Data quality assurance

#### 4.9.1. Pre analytical

All biopsy samples were processed at once using "Tissue Tek II" VIP processing machine.

All reagents for tissue processing; formaldehyde, alcohol, xylene and paraffin wax were all new or freshly prepared.

All the staining reagents were prepared by standard procedures as shown in the annex.

Expiration date was checked for every powder or solution that has been used in the preparation of reagents.

Embedding, sectioning, and labeling were done by experienced laboratory technologists, histotechnologists and the PI.

#### 4.9.2. Analytical

Both liquid dish washing soaps used Master® and Sine® have been cleared by Ethiopian Conformity Assessment Enterprise (ECAE)

Standard staining procedures were followed for staining the sections.

Both conventional and XAF H&E staining procedures were performed by the PI.

#### 4.9.3. Post analytical

All the pathologists were blinded.

All the three pathologists are experienced pathologists.

All the data were registered and entered to IBM SPSS version 24 by the PI.

### 4.10. Statistical analysis

Data was entered into IBM SPSS version 24 for analysis.

McNemar's test was performed to show the equivalence/agreement between the matched paired sections (conventional and XAF H&E stained sections), $p<0.05$ considered as statistically significant.

Most similar researches used "*Z*" value to look for proportion difference between the two methods stained section. Therefore, in order to compare our research results with those researches, "*Z*" test was used, $p<0.05$ considered as statistically significant.

### 4.11. Ethical consideration

Ethical clearance was obtained from departmental research ethics review committee (DRERC) of the department of pathology, collage of health science, Addis Ababa University. The data from study sample was used for the purpose of this research only. Numerical codes were used all the time. Hence, anonymity and confidentiality were strictly maintained.

## 5. RESULTS

To compare the deparaffinization ability of dilute liquid dish washing soap with xylene in hematoxyline and eosin staining, 141 matched pairs of biopsy slides were included in this study. All the slides were evaluated by three pathologists independently. All pathologists used a checklist that contains five parameters. From the five parameters, those slides that only fulfilled the three or more parameters were considered adequate for diagnosis.

Both Pathologist I and II reported 139(98.6%) conventionally stained sections, adequate for nuclear staining while 119(84.4%) and 138(97.9%) of XAF stained sections were adequate, respectively. Pathologist III reported 124(87.9%) of XAF and all conventionally stained sections as adequate for nuclear stain. From 141 pair of slides each pathologist evaluated, disagreement reported in 22, 3 and 17 pairs as reported by pathologist I, II and III respectively. All pathologists favored conventionally stained sections over XAF stained sections. Both "*Z*" and MacNemar's test indicated no significant difference between the methods as pathologist II while pathologist I and III showed significant difference (table 2).

**Table 2:** Nuclear staining of the matched pairs of conventional and XAF stained sections

| Nuclear staining | | | | |
|---|---|---|---|---|
| **XAF** | **Conventional** | | | **"Z" value & McNemar's *p* value** |
| **Pathologist 1** | Inadequate | Adequate | Total | |
| Inadequate | 1 | 21 | 22 | *Z*=4.3, *p*<0.05 |
| Adequate | 1 | 118 | 119 | McNemar's *p* value = 0.000 |
| Total | 2 | 139 | 141 | |
| **Pathologist 2** | | | | |
| Inadequate | 1 | 2 | 3 | *Z*=0.5, *p*>0.05 |
| Adequate | 1 | 137 | 138 | McNemar's *p* value= 1.000 |
| Total | 2 | 139 | 141 | |
| **Pathologist 3** | | | | |
| Inadequate | 0 | 17 | 17 | *Z*=4.3, *p*<0.05 |
| Adequate | 0 | 124 | 124 | McNemar's *p* value = 0.000 |
| Total | 0 | 141 | 141 | |

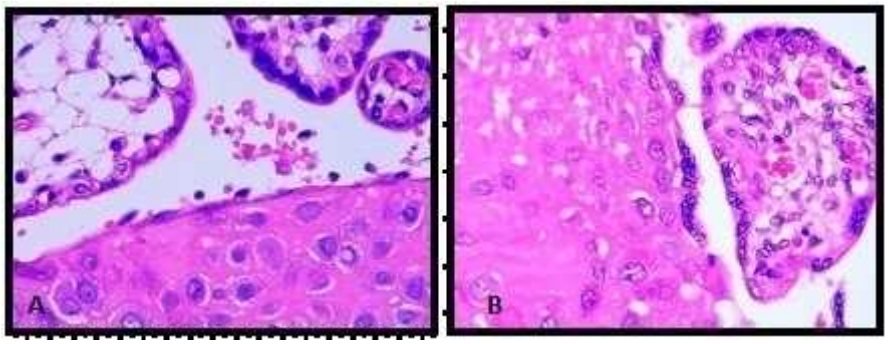


**Figure 2: Comparison of xylene-alcohol free and conventional H&E stained histological sections. Photomicrographs showing adequately stained deciduas and chorionic villi**

**(A) conventional, 4x (B) Xylene-alcohol free, 4x**

For cytoplasmic staining, 119 (84.4%) of XAF and 139 (98.6%) of conventionally stained section were reported as "adequate" by pathologist I, indicating statistically significant difference between the methods. Pathologist II reported 139(98.6%) of XAF and 140(99.3%) of conventionally stained sections for cytoplasmic stain adequacy. Pathologist III showed 133

(94.3%) of XAF & 129 (91.5%) of conventionally stained section were adequate in cytoplasmic stain. Pathologist I, II and III showed discrepancy in 22, 1 and 14 pair of slides respectively. Pathologist II and III report indicated no significant difference between the methods while Pathologist III favored XAF stained sections as shown on table 3.

**Table 3:** Cytoplasmic staining of the matched pairs of conventional and XAF stained sections

| **Cytoplasmic staining** | | | | |
|---|---|---|---|---|
| **XAF** | **Conventional** | | | **"*Z*" value & McNemar's *p* value** |
| **Pathologist 1** | Inadequate | Adequate | Total | |
| Inadequate | 1 | 21 | 22 | *Z*=4.3, *p*<0.05 |
| Adequate | 1 | 118 | 119 | McNemar's *p* value= |
| Total | 2 | 139 | 141 | 0.000 |
| **Pathologist 2** | | | | |
| Inadequate | 1 | 1 | 2 | *Z*=0.6, *p*>0.05 |
| Adequate | 0 | 139 | 139 | McNemar's *p* value |
| Total | 1 | 140 | 141 | =1.000 |
| **Pathologist 3** | | | | |
| Inadequate | 3 | 5 | 8 | *Z*=0.9, *p*>0.05 |
| Adequate | 9 | 124 | 133 | McNemar's *p* value |
| Total | 12 | 129 | 141 | =0.424 |

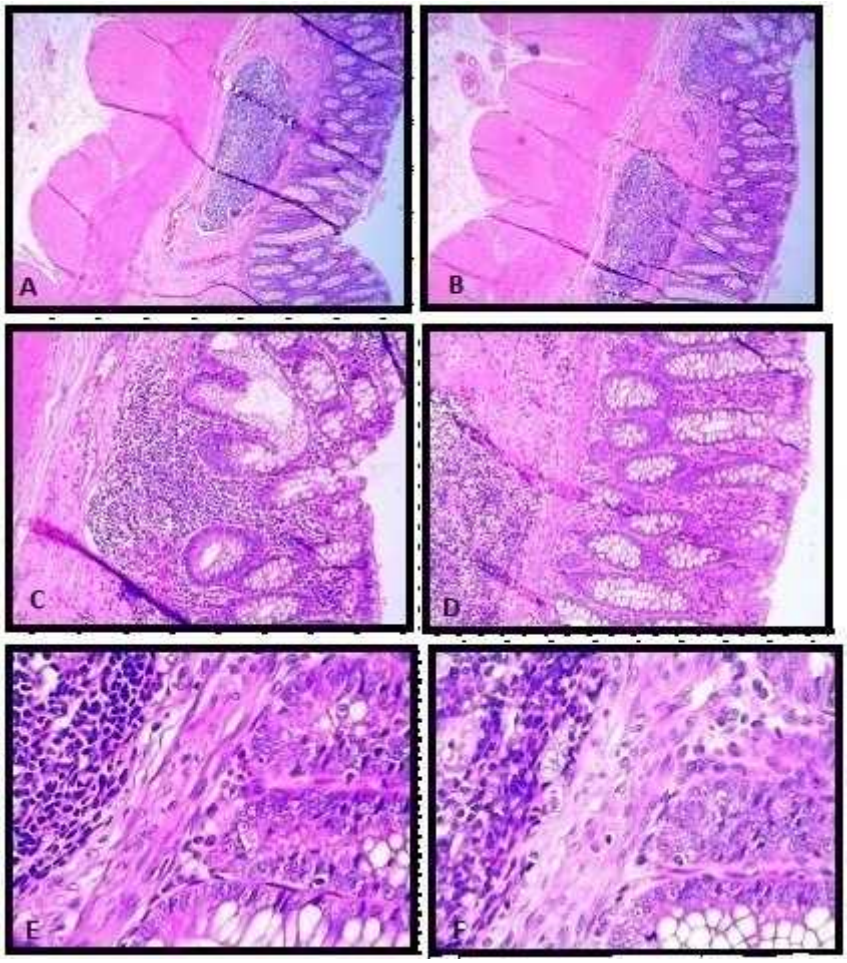


**Figure 3: Comparison of xylene-alcohol free and conventional H&E stained histological sections of colonic biopsy specimen. Photomicrographs showing adequate nuclear, cytoplasmic stain; clarity and crispiness in the mucosal as well as smooth muscle**

**(A) 4x (C) 10x and (E) 40x are conventional sections**

**(B) 4x (D) 10x and (F) 40x are Xylene-alcohol free sections**

Both pathologists (I&II) reported 137 (97.2%) of conventionally stained section as clear, while 116 (82.3%) & 124 (87.9%) of XAF stained slides showed clarity respectively. From 141 pair of stained sections, pathologist I and II reported 23 and 13 untied pairs respectively while pathologist III showed no disagreement between the methods for clarity of staining. Significant difference between the two methods on clarity is indicated by pathologist I & II (Table 4).

**Table 4:** Carity of the matched pairs of conventional and XAF stained sections

| | **Clarity of staining** | | | |
|---|---|---|---|---|
| | **Conventional** | | | **"*Z*"value & McNemar's *p* value** |
| **XAF** | Absent | Present | total | |
| Pathologist 1 | | | | |
| Absent | 3 | 22 | 25 | *Z*=4.1, *p* <0.05 |
| Present | 1 | 115 | 116 | McNemar's *p* value = 0.000 |
| Total | 4 | 137 | 141 | |
| Pathologist 2 | | | | |
| Absent | 4 | 13 | 17 | *Z*=3, *p* <0.05 |
| Present | 0 | 124 | 124 | McNemar's *p* value = 0.000 |
| Total | 4 | 137 | 141 | |
| Pathologist 3 | | | | |
| Absent | 0 | 0 | 0 | *Z*=0, *p* >0.05 |
| Present | 0 | 141 | 141 | McNemar's *p* value = 1.000 |
| Total | 0 | 141 | 141 | |

Pathologist I reported 48 untied pairs for uniformity of staining that revealed a significant difference between the methods. Uniformity was present in 131 (92.9%) conventionally stained sections while only 93(66%) XAF stained sections were uniform according to pathologist I. Pathologist II noted that 139 (99.3%) of both conventional and XAF stained slides were uniform, with only 2 unmatched pairs. Pathologist III reported 121(85.8%) of XAF and 123(87.2%) of conventionally stained sections as uniform with 26 section pairs discrepancy. Therefore, according to both pathologists, there was no difference between the two methods as shown on table 5.

**Table 5:** Uniformity of the matched pairs of conventional and XAF stained sections

| Uniformity of staining | | | | |
|---|---|---|---|---|
| **XAF** | **Conventional** | | | **"Z" value & McNemar's *p* value** |
| | Absent | Present | Total | |
| **Pathologist 1** | | | | |
| Absent | 5 | 43 | 48 | *Z*=5.6, *p* <0.05 |
| Present | 5 | 88 | 93 | McNemar's *p* value= 0.000 |
| Total | 10 | 131 | 141 | |
| **Pathologist 2** | | | | |
| Absent | 0 | 1 | 1 | |
| Present | 1 | 139 | 140 | *Z*=0, *p*>0.05 |
| Total | 1 | 140 | 141 | McNemar's *p* value= 1.000 |
| **Pathologist 3** | | | | |
| Absent | 6 | 14 | 20 | *Z*=0.3, *p*>0.05 |
| Present | 12 | 109 | 121 | McNemar's *p* value= 0.845 |
| Total | 18 | 123 | 141 | |

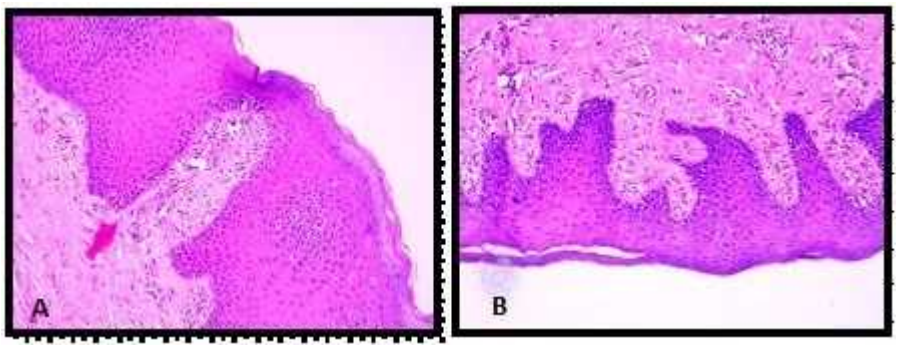


**Figure 4: Comparison of xylene-alcohol free and conventional H&E stained histological sections. Photomicrographs showing adequately stained stratified squamous epithelium,**

**(A) conventional H&E, 4x (B) Xylene-alcohol free H&E, 4x**

Pathologist I reported 103 (73%) conventional and 90 (66%) XAF stained sections as crisp while 21 pair of sections disagreed. Crispiness of staining showed disagreement in 54 pair of slides while 131 (92.9%) conventional and 87(61.7%) XAF stained sections were crisp as pathologist II. Pathologist III reported 137 (97.2%) slides as crisp for both methods. McNemar's test showed

agreement between the two methods for crispiness of staining as reported by pathologist III. But *Z* test of proportion indicated no significant difference between the two methods for crispiness of staining as Pathologist I and III, shown on the table 6.

**Table 6:** Crispiness of the matched pairs of conventional and XAF stained sections

| **Crispiness of staining** | | | | |
|---|---|---|---|---|
| | **Conventional** | | | |
| **XAF** | Absent | Present | Total | **"*Z*"value & McNemar's *p* value** |
| **Pathologist 1** | | | | |
| Absent | 34 | 17 | 51 | *Z*=1.7, *p* >0.05 |
| Present | 4 | 86 | 90 | |
| Total | 38 | 103 | 141 | McNemar's *p* value= 0.007 |
| **Pathologist 2** | | | | |
| Absent | 5 | 49 | 54 | *Z*=6.3, *p* <0.05 |
| Present | 5 | 82 | 87 | |
| Total | 10 | 131 | 141 | McNemar's *p* value = 0.000 |
| **Pathologist 3** | | | | |
| Absent | 0 | 4 | 4 | *Z*=0, *p* >0.05 |
| Present | 4 | 133 | 137 | |
| Total | 4 | 137 | 141 | McNemar's *p* value = 1.000 |

Pathologist I report showed 138(97.9%) of conventional and 116(82.3%) of XAF stained sections were diagnosable with 22 untied pairs of sections. Both Pathologist II and III reported 140(99.3%) conventionally stained sections were diagnosable while 138(97.9%) and 139(98.6%) of XAF stained sections were diagnosable respectively. Pathologist II and III result indicated no difference between the methods for diagnosis by showing a good agreement for the paired sections as shown on table 7.

**Table 7:** Total score of the matched pairs of conventional and XAF stained sections for diagnosis

| XAF | Diagnosis Conventional | | | "Z" value & McNemar's *p* value |
|---|---|---|---|---|
| | Inadequate | Adequate | Total | |
| **Pathologist 1** | | | | |
| Inadequate | 3 | 22 | 25 | *Z*=4.4, *p* <0.05 |
| Adequate | 0 | 116 | 116 | McNemar's *p* value= 0.000 |
| Total | 3 | 138 | 141 | |
| **Pathologist 2** | | | | |
| Inadequate | 1 | 2 | 3 | *Z*=1, *p* >0.05 |
| Adequate | 0 | 138 | 138 | McNemar's *p* value = 0.500 |
| Total | 1 | 140 | 141 | |
| **Pathologist 3** | | | | |
| Inadequate | 0 | 2 | 2 | *Z*=0.6, *p* >0.05 |
| Adequate | 1 | 138 | 139 | McNemar's *p* value = 1.000 |
| Total | 1 | 140 | 141 | |

To compare the two methods; McNemar's and "*Z*" test were used. Both methods showed consistent agreement to one another for each parameter expect on crispiness of staining for pathologist I. When "*Z*" test value (*Z*=1.7, *p*>0.05) showed no significant difference McNemar's *p* value (0.007) indicated significant difference between the methods. This is because when "*Z*" test simply compares the proportion between the methods McNemar's test compare the matched pairs. So this difference implies many slides that have been reported inadequate in XAF slides were adequate in conventional method and vice versa. This is one of the reason this research has used more than one analysis tool to compare the two methods.

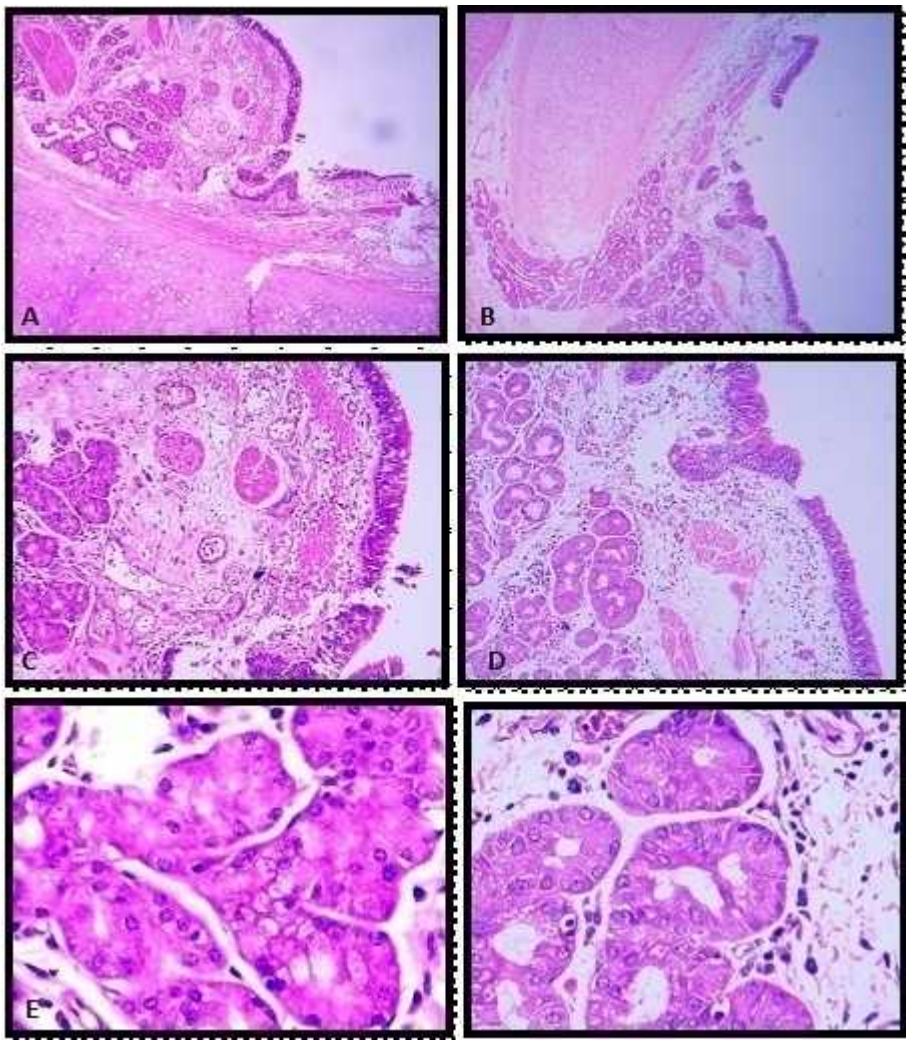


**Figure 5: Comparison of xylene-alcohol free and conventional H&E stained histological sections of bronchial biopsy specimen. Photomicrographs showing adequate nuclear and cytoplasmic stain with crisp border and clarity in the respiratory epithelium; mucinous gland and adjacent cartilage,**

**(A) 4x (C) 10x and (E) 40x are Xylene-alcohol free sections**

**(B) 4x (D) 10x and (F) 40x are conventional sections**

# 6. DISCUSSION

In this study, liquid dish washing soap was used as a substitute to xylene for staining biopsy sections and compared with the routine H&E staining procedure. Personal preference variation between the three pathologists was significant in this study. Therefore, the results of each pathologist were analyzed and discussed separately. All results that show significant difference between the methods favor conventional stained sections over XAF stained pairs.

Pathologist II reported no significant difference between the two methods stained sections for nuclear stain and this agrees well with many researches [18-24]. In contrary, pathologists I & III reports indicated a significant difference between conventional and XAF staining methods on nuclear stain in this study. Some of the studies mentioned the possible causes of nuclear stain inadequacy. Those causes could be: incomplete deparaffinization, inadequate staining time, contaminants in rinsing solution or insufficient pre rinsing with water prior to staining with hematoxylin[18,19,23]. The experiences that were gained in the pilot studies and the precautions taken to avoid the commonly known potential causes of nuclear staining inadequacy including the suggestions from other studies made the finial slides of this research better (refer figure 2-5). After the pathologists report, a relatively better nuclear staining was seen by staining the XAF sections in the hematoxyline longer time than the routine procedure. We suppose similar to staining time, concentration of the hematoxyline might be the cause for XAF sections weak nuclear staining in this study. This has been said due to the fact that most of the above studies used Harris hematoxyline while Mayer's hematoxyline was used in this study.

Pathologist I and III report indicated no significant difference between the two methods stained sections regarding crispiness; this is supported by several studies [20-24]. In contrary, pathologist II showed a statistically significant difference between the methods like other studies [18,19] but here pathologist II favored conventional sections unlike the two studies. In these two studies they said Mayer's hematoxylin gives XAF stained section ideal crispiness. This is opposite to our finding. But this contradiction of the result might not be due to the staining method difference for the staining parameter "crispiness" since personal preference variation between the pathologists is significant here. According to Pathologist III the paired sections showed the same and higher proportion (97.2%) for crispiness. Pathologist II also reported

higher proportion (92.9%) of conventionally stained sections as crisp but significantly lower proportion (61.7%) for XAF stained sections. On the other hand, as pathologist I, crisp sections were significantly lower in number for both conventional (73%) and XAF (63.8%) stained slides.

Pathologist II and III showed that there was no difference between the two methods stained sections for cytoplasmic stain like many similar studies [20,21,23,24] where as pathologist I report indicated a statistically significant difference between the slides like some other researches [18,19]. Most of the above researches [20,21,23,24] used harris hematoxyline for both XAF and conventional sections. Since it is a regressive staining, it needs differentiation to avoid hematin interference with the eosin stain. However, sections showed bluish cytoplasmic stain. So they solved the problem by increasing the eosin staining time for 30 seconds more. In other studies, where they used Mayer's hematoxyline, the cytoplasmic stain was compromised due to the alkalinity of the bluing agent (Lithium carbonate) and tap water [18,19]. If alkaline bluing agent is inappropriately washed before eosin staining or the tap water used after eosin staining is alkaline, the acidic stain will be washed out resulting weak cytoplasmic stain [18,19]. In our case as Mayer's hematoxylin was used, differentiation step was not needed and no bluing agent other than water was used. But the pilot findings indicated, from the paired slides XAF section was more sensitive for concentration and time in eosin stain. Adding the staining time in eosin, improved the cytoplasmic staining of the section observably. More than the strong agreement pathologist II showed, report from pathologist III, that favored XAF stained slides (94.3%) over conventionally stained slides (91.5%) on cytoplasmic staining is a proof for the improved quality (refer figure 2-5).

Pathologist I&II report showed significant difference between the methods favoring conventionally stained sections on clarity of staining. But pathologist III reported all slides (100%) from both staining methods as clear, the same as a study done by Anuradha A. *et al.*, in 2014 [22].

As far as uniformity of staining is concerned, Pathologist II (99.3% uniform, for both method stained slides) and Pathologist III (87.2% for conventional and 85.8% for XAF stained slides as uniform) report indicated a strong agreement between the methods on uniformity of staining which is similar to some previous studies [21,22,24]. But pathologist I showed a significant

difference between the methods in favor of conventionally stained sections, similar to other studies [18-20,23].

Patchy staining and cloudy areas in XAF stained sections downgraded the clarity and uniformity of the staining pattern in this study. Similar studies mentioned that, this errors could be due to tear, rip or fold in sections, thick sections, moisture on cover slip, dirty microscopic lenses, introduction of extraneous tissue, unclean blade or improper deparaffinization [18-20]. For the improper deparaffinization (dewaxing) problem: Maintaining the DWS temperature exactly at $90^0$c(not less), keeping the slides for no less time than the recommended in the DWS/ $dH_2O$ and adding another liquid DWS (DWS III for 30 second at 90°C) in the staining protocol were mentioned as a remedy for the clarity and uniformity problem[18-20]. And from their suggestion and the pilots result, by increasing the concentration of the DWS to 2%, by using digital water bath to maintain the temperature of the DWS and by keeping the sections for 2 min in each DWS and 1 min in each distilled water, it was able to bring a better result on clarity and uniformity of the staining. Results reported by pathologist III for clarity and Pathologist II & III for uniformity of staining is a proof (refer figure 2-5). Furthermore, from the pilot studies experience, freshly preparing the 2% DWS found to be very important for the quality of the staining in XAF sections. Finally, simple sectioning errors like folds that don't normally interfere on conventional sections happened to have noticeable impact on the quality of the XAF sections as we witnessed.

A variety of tissues were used in this study, yet no possible pattern could be found between the above five parameters and tissue types. In general, the pathologists saw some sort of inadequacy on one or more of the parameters in XAF stained slides on Psammomatous meningioma (brain tumor biopsy), bone marrow, transitional epithelium (bladder biopsy) and abscess cavity (breast biopsy) sections. From the above biopsies, the most compromised were the brain tumor and bone marrow. These biopsies were surface decalcified by 5% hydrochloric acid [3]. Though decalcification is known to compromise staining quality in general, its effect is pronounced in XAF sections in this study. This is an open area to be uncovered in further studies.

With regard to diagnosis, the report from pathologist I showed statistically significant difference between the methods. On the other hand, report from pathologist II and III showed significant agreement between the matched paired sections like previous studies [1,11,18-23].

Results of similar previous studies have shown certain differences between the methods regarding the various staining parameters used in this study. However, all studies concluded that the XAF H&E stained sections (staining method) are similar to the conventionally stained H&E section (staining method) [1,11,18-24]. Even though they are similar, the XAF method is more advantageous than the conventional method for it is being nontoxic, economical, nonflammable, nonhazardous and easy to dispose. Economically, in H&E staining procedure for conventional method, xylene and alcohol consumption costs 9.61 birr per slide while in XAF procedure DWS consumption costs 0.054 birr per slide. This shows that using the XAF method for section deparaffinization is 178 times cheaper than the conventional method.

# 7. STRENGTH AND LIMITATION OF THE STUDY

## 7.1 Strength of the study

The sample size was large as compared to previous related researches.

Most of the related studies used one pathologist for slide evaluation while this research used three pathologists

Seven pilot studies were carried out in this research, using two, ECAE cleared liquid dish washing soaps (Master® and Sine®)

## 7.2 limitation of the study

Different kinds of hematoxylin stains were not tried for nuclear staining in XAF procedure.

Using more pathologists could have solved the personal variation problems faced in this research

The quality of the microscope each pathologists used were not assessed

# 8. CONCLUSION AND RECOMMENDATION

## 8.1 Conclusion

In this study, among the three pathologists, two of them showed that by using XAF stained sections, adequate diagnosis can be made. Generally replacing Xylene and alcohol with DWS has a great advantage in relation to economy, 178 times cheaper. Besides, eliminating xylene brings about a better working environment in the laboratory. Due to the simplicity of disposal system, it creates a better environment for the general population at large. It also simplifies laboratory ventilation requirements and permits greater flexibility in laboratory design/location.

Therefore, considering the advantages of the new method with regard to the pathologists report in this study, it is possible to say that XAF H&E staining method meets today's needs of histopathology laboratory at large.

## 8.2 Recommendation

I recommend another study by including more pathologists for evaluation with different kind of hematoxylin and eosin stain and staining approach using different kind of liquid dish washing soap

It is useful to expand the study by including special stain and immunohistochemistry

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## Annex I: Conventional hematoxylin and eosin staining procedure

**Depparafinization and rehydration:**

Xylene I 5min

Xylene II 5min

100% alcohol 5 min

90% alcohol 5min

70% alcohol 5min

Water wash 10min

**Nuclear Staining**

Mayer's haematoxylin 10min

Bluing 10min

**Cytoplasmic Staining**

1% Eosin 1min

Dehydration 70% alchohol 30 sec

90% alcohol 30 sec

Absolute alcohol 30sec

Xylene I 5 min

Xylene II 5 Min

**Total time=69 min**

## Annex II: Xylene and Alcohol free hematoxylin and eosin staining method

**Deparaffinisation**

2% diluted liquid dishwashing detergent-I At $90^0$C 2 min

2% diluted liquid dishwashing detergent-II At $90^0$C 2 min

Distilled water-I At $90^0$C 1 min

Distilled water-II At $90^0$C 1 min

Wash slides in distilled water At $45^0$C 30sec

Wash slides in distilled water At room temperature 30sec

**Nuclear Staining**

Mayer's Haematoxylin At room temperature 10 min

Tepid water wash 4 min

Bluing At room temperature 10 min

**Cytoplasmic staining**

Eosin 1% At room temperature1 $1^{1/2}$ min

Wash slides in distilled water1 min

Over drying the sections At $60^0$C 10 min

**Total time=43 min**

## Annex III: Processing schedule

| No | Reagents | Time | Temperature |
|---|---|---|---|
| 1 | 4% Formalin | 1 hour | $35^0$c |
| 2 | Water | 5 second | $35^0$c |
| 3 | 50% Ethanol alcohol | 1 hour | $35^0$c |
| 4 | 70% Ethanol alcohol | 1 hour | $35^0$c |
| 5 | 80% Ethanol alcohol | 1 hour | $35^0$c |
| 6 | 90% Ethanol alcohol | 1 hour | $35^0$c |
| 7 | 100% Ethanol alcohol | 1 hour | $35^0$c |
| 8 | 100% Ethanol alcohol | 1 hour | $35^0$c |
| 9 | Xylene | 1 hour | $35^0$c |
| 10 | Xylene | 1 hour | $35^0$c |
| 11 | Paraffin wax | 45 minutes | $68^0$c |
| 12 | Paraffin wax | 45 minutes | $68^0$c |
| 13 | Paraffin wax | 45 minutes | $68^0$c |
| 14 | Paraffin wax | 45 minutes | $68^0$c |

## Annex IV: Pathologist Checklist

H and E stained section grading parameters:

- Nuclear staining (adequate = score 1, inadequate = score 0)
- Cytoplasmic staining (adequate = score 1, inadequate = score 0)
- Clarity of staining (present = score 1, absent = score 0)
- Uniformity of staining (present = score 1, absent = score 0)
- Crispness of staining (present = score 1, absent = score 0).

| Slide lable/code | Nuclear staining | Cytoplasmic staining | Clarity of staining | Uniformity of staining | Crispness of staining |
|---|---|---|---|---|---|
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## Annex V: Investigator Check list

| Biopsy No | R.code and label conventional | R.code and label DWS |
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## Annex VI: Hematoxyline and eosin preparation

**Mayer's hematoxylin**

Preparation of solution

Hematoxylin 1 g

Distilled water 1000 ml

Potassium or ammonium alum 50 g

Sodium iodate 0.2 g

Citric acid 1 g

Chloral hydrate SLR 50 g or

Chloral hydrate AR 30 g

The hematoxylin, potassium alum, and sodium iodate are dissolved in the distilled water by warming and stirring, or by allowing to stand at room temperature overnight. The chloral hydrate and citric acid are added, and the mixture is boiled for 5 minutes, then cooled and filtered. If higher-purity chloral hydrate AR grade is used, the amount may be reduced, as shown above. The stain is ready for use immediately. Filter before use.

**1% Eosin**

Eosin Y 10 gm

Distilled water 1000ml

Acetic acid 0.5 ml

A crystal of thymol can be added to inhibit the growth of fungi.

## ANNEX VII: Declaration

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Dollar Nureddin: _____ Date: _____

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