Addis Ababa University
School of Medicine
Post Graduate Program
Pathology Department

Evaluating Efficacy of paraffin oil, Isopropanol and ethanol mixture as alternative for Xylene in surgical biopsy tissue processing by the conventional paraffin wax method: comparative study in histopathology

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A thesis submitted to the School of Graduate Studies, Addis Ababa University, School of medicine, pathology department in partial Fulfillment of the Requirements for the Degree of Master of Science in Histopathology.

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This is to certify that the thesis prepared by Natinael Berhane, entitled: **Evaluating Efficacy of PIE mixture (mixture of paraffin oil, Isopropanol and ethanol) as alternative for Xylene in surgical biopsy tissue processing by the conventional paraffin wax method: Comparative study in histopathology** and submitted in fulfillment of the requirements for the Degree in Master of Science (Histopathology) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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Acknowledgement

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Abbreviations

AAU	Addis Ababa university
ACSH	Ayder comprehensive specialized hospital
ATSDR	Agency for Toxic Substances and Disease Registry
CIM	Clearing and Infiltrating Mixture
CO	Coconut oil
COVID-19	Corona Virus disease -2019
CXM	Conventional Xylene Method
DNA	Deoxyribonucleic acid
DPX	Di-N-Butyle Phthalate in Xylene
DWS	Dish washing soap
EFMHACA	Ethiopian Food, Medicine, Healthcare Administration & Control Authority
EPA	Environmental Protection Agency
FHRH	Felege-Hiwot Referral Hospital
FMoH	Federal Ministry of Health
GO	Groundnut oil
Hand E	Hematoxylin and Eosin stain
HURH	Hawassa University Referral Hospital
ILO	International Labor Organization
NCCLS	National Committee for Clinical Laboratory Standard
^{O}C	Degree centigrade.

OSHA	Occupational Safety and Health Authority
PI	Principal Investigator
PIE	Paraffin oil, Isopropanol, and Ethanol mixture
PPM	Particles per Minute
RHB	Regional Health Bureaus
RIs	Refractive index
SPHMMC	St. Paul's Hospital Millennium Medical College
SoM	School of Medicine
SOP	Standard Operating Procedure
SPSS	Statistical Package for Social Science
TASH	Tikur Anbesa specialized hospital
TAT	Turnaround Time
TWA	Time-Weighted Average
VOCs	Volatile Organic Compounds

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Operational Terminologies

Biopsy:-A surgical procedure in which a small piece of tissue is removed from a patient for histopathology investigation.

Bits –small slices of representative tissues taken from the biopsy for processing.

Blocks: cassettes filled with paraffin wax holding infiltrated and embedded tissues in side

Cassettes: small plastic rectangular sieve like materials used for keeping the tissue inside duringtissue processing and embedding.

Clarity of staining: devoid of cloudiness throughout the section.

Clearing-The step following dehydration is called "clearing" and consists of replacing the dehydrants with a substance that will be miscible with the embedding medium (paraffin). As a result,when the tissue is completely infiltrated with the clearing agent, it becomes translucent.

Crispiness of staining: being able to see well delineated nuclear membranes and sharply stained condensed chromatin against an unstained nucleolus.

Cytoplasmic staining: the intensity of eosinophilia in a cell.

Dehydration: removal of water from the tissue.

Fixation: - is the process of keeping the tissue or cells as life state as possible by the use of fixative solution. The use of a fixative is to preserve histological or cytological specimens.

Gross examination: - Macroscopic study of tissue changes.

Grossing: describing tissues macroscopically dimensions and taking representative samples. **Histopathology**: - a branch of pathology concerned with the tissue changes characteristic of disease.**Impregnation**: - The process of diffusing or permeating with another substance,

Mounting: - to prepare (a sample) for examination by a microscope, as by covering the slide bycoverslip.

Nuclear staining: the intensity of basophilia in a cell.

Productivity: rate of output compared toinput.in histopathology it is the number of patients (samples) that a personnel can serve with in given working time.

Sectioning (Microtomy):-preparation of thin sections from fixed and embedded tissue blocks.
Staining:-artificial coloration of a substance to facilitate examination of tissues, microorganisms orother cells under the microscope.

Tissue Processor: - The machine uses to process animal and human tissues automatically. It useschemicals like Formalin, alcohol, Xylene and Paraffin.

Turnaround Time (TAT):- The interval between the ordering of a clinical laboratory test or otherdiagnostic procedure and the reporting of results.

Uniformity of staining: devoid of patchy staining and out-of-focus areas throughout the section.

Abstract

Background: *Major global mortality is due to non-communicable diseases with cancer predominance. Unfortunately, Cancers diagnosed at late to end stages in Africa. Histopathology diagnosis has tremendous value in the treatment, prognosis and follow up of cancer patients. Fixation, processing, embedding, sectioning, staining and evaluation of slides are mandatory procedures in histopathology. These procedures use noxious and carcinogenic chemicals primarily xylene. However; xylene is hazardous chemical that causes muscular, neuron, ocular, reproductive, respiratory, immunological, hematological, renal, hepatic, cardiac effects. Fluid and vapors of xylene cannot be prevented with glove and masks. Hence there should be some substitution mechanism which can minimize or replace use of xylene in histopathology laboratory.*

Objective*: This study aimed to evaluate the comparative efficacy of paraffin oil, isopropanol and ethanol mixture (PIE) as a bio friendly replacement for noxious xylene in surgical tissue processing with conventional paraffin wax method.*

Method: *Prospective cross-sectional method and purposeful sampling technique was used at St. Paul's hospital millennium medical college pathology department from February 2019 up to December 2020 GC. A total of 112 samples were processed using new mixture and conventional xylene method. Block sectioning simplicity and slide quality for diagnosis were evaluated by histologists and pathologists using standard checklist respectively.*

Result: *According to the study**,** report of the three histologists revealed that 94.4% and 94.5% blocks were easy to produce good sections with paraffin oil, isopropanol and ethanol mixture and Conventional xylene methods respectively; indicating no statistical difference among methods (p=0.967). Evaluation of mounting, block storage stability and physical quality of sections confirmed adequacy in 98.2%, 99.1% and 98.3% respectively. This result showed no statistical significant difference with the conventional method (p=1.000)). The new xylene free method showed 95 % and 96.4% nuclear and cytoplasmic staining adequacy respectively.no statistical difference in both parameters recorded (p=0.602 and0.772 respectively) 95.3%, 96.8% and 95.5 % of stained slides which were processed with the new method revealed adequacy for clarity, uniformity and crispness of staining respectively indicating no statistical significant difference as p=0.073, 0.108, and 0.180 respectively. About 95.8% of total slides were adequate for diagnosis with the new method which was not statistically significant (p=0.668& p= 0.229 by score and observant respectively).*

Conclusion: *The new mixture has comparable ability with that of xylene to clear surgical biopsy specimens during processing in histopathology.*

Keywords: clearing, health hazardous, PIE mixture, Pathology, substitute, tissue processing.

1 INTRODUCTION

1.1 Background

Major global mortality is due to non-communicable diseases with cancer predominance [1]. In Africa, cancer is emerging as critical public health problems [2].Trends show that even though incidence rate is half of western world, death is similar with Africa [3].Of all cancers, prostate cancer in men and breast cancer in women were the commonest ones in Africa [4].In Ethiopia cancer holds about 5.8% of the total national mortality from these 83% were late diagnosed [5].

Unfortunately nearly all patients in Africa with cancers diagnosed at late to end stage [6]. Chronic diseases especially Cancer diagnosis and treatment strongly dependent on report of histopathology laboratories which receive surgical biopsy samples and do a lot of tissue techniques following grossing. These include tissue preparation by variety of chemicals (formalin, ethanol, xylene, and paraffin wax) paraffin embedding, sectioning with microtome, and hematoxylin and eosin (H&E) staining followed by slide interpretation under microscope [7].

Quality slide production emanates from perfect histopathology procedures which provide visualization of tissues and micro structural changes of cells under study [8]. Fundamentally desirable sectioning of tissues needs treatment of tissues through impregnation in paraffin medium [9]. Standardization and proper implementation of all procedures are vital as evaluation is done through comparison of diseased or experimentally altered with healthy or control tissues [10].

The longest and major histopathology procedure is tissue processing. Routine manual, rapid manual, and microwave methods are the three common types. Fixation, dehydration, clearing and impregnation are the steps in it [11].

Clearing is the step following dehydration which is replacing of the dehydrants with a substance that will be miscible with the embedding medium (paraffin). As a result, when the tissue is completely filled with the clearing agent, it becomes more transparent. Tissue clearing techniques all aim to make tissues or cell cultures more transparent to overcome their opacity.

Opacity prevents them from being penetrated by visible wavelengths of light under microscope. Histopathology laboratories always faced with opaque biological tissues [11, 12].

Biological tissues have many organic and inorganic molecules. Most biological tissues are composed of water (70–80%) with low RI (n = 1.33), proteins (∼10%) with high RI (n > 1.44), and lipids (∼10%) with high RI (n > 1.45). The difference (heterogeneity) of tissue components in optical property likes refractive index (RI) and light absorption provides them opacity. Heterogeneity either scatters or decays light by absorption [13]. Scatter and decay of incoming light should be eliminated for the purpose of microscopic evaluation of tissues. This mandates tissue clearing in histopathology procedures. The purpose of tissue clearing is to eliminate the opacity nature of tissue and make it transparent for its suitability in the intended use.

Tissue clearing methods usually approached either by replacement of liquids & mixtures with high RIs or homogenizing the RIs [14].RIs homogenization of tissue molecules should be done without loss or disruption of intended ones. Tissue clearing methods usually approached either by replacement of liquids & mixtures with high RIs or homogenizing the RIs [14].RIs homogenization of tissue molecules should be done without loss or disruption of intended ones. The major tissue molecules are water, lipid and protein. The RIs of these molecules should be homogenized for clearing to happen. Naturally, RI of water is much less than tissue protein. Hence, it has to be substituted with a high RI medium in the homogenization process of RI whereas Lipid has high RI and causes scattering of light. Therefore; either removal of lipids or lowering RI is important in homogenization of lipid [15, 16, and 17].

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Permeability of the plasma membrane is mandatory for the reagents exchange during clearing. This needs permeabilization of cells. Permeabilization removes more cellular membrane lipids to allow large molecules to get inside the cell. It promotes substitution of water with high RI medium through osmotic pressure at high concentration. It also advances removal of lipids to facilitate passive diffusion of external molecules. Reagents used for this purpose may be water-miscible polar solvents, hyper hydration reagents with/without dilapidation reagents **[18]**.

Tissue clearing can be done using various organic and inorganic solvents. Miscibility, refractive index, polarity and toxic nature of the chemicals used for clearing (which perform permeabilization and homogenization) are basic criteria in selecting reagents. For instance, when xylene is used for clearing; dehydration and permeabilization are done via alcohol or ether treatments. The miscibility nature of aromatic solvents with organic solvents allows high RI aromatic solvents (n ~ 1.56) to homogeneously fill inside the tissue and provides clearing**[19, 20]**.

Since 1950s, xylene is being used in histopathology laboratory; in tissue processing, slide staining and mounting steps [21, 22].These procedures take more than a day; therefore histopathology personnel are highly breathing xylene vapor. As a result, tremendous health damages are known due to xylene **[23]**. There is high Exposure of pathology laboratory personnel's to numerous potentially hazardous chemicals. Xylene is the most usual chemical in histopathology laboratory [24].

Safe and healthy working environment is a fundamental human right of all working people in the world **[25]**. Based on this fact there need a way to create such working environments in the laboratories. Primarily substitutions of xylene, otherwise local exhaust ventilation or protective equipment usage are mandatory in these areas due to xylenes potential toxicity and flammability nature [26].

Comparison of the physical and chemical property of xylene with that of paraffin oil, isopropanol and ethanol mixture gives significant safety and occupational hazard improvements. Paraffin oil is non-polar compound while ethanol and isopropanol are polar compounds. At 20^{o} c the refractive index of paraffin oil, isopropanol, xylene and tissue water is 1.468, 1.377, 1.50 and 1.33 respectively. The density of paraffin oil is 0.82 – 0.88gm/ml as compared to 0.79gm/ml of isopropanol and 0.86gm/ml of xylene. Comparatively, isopropanol has very limited health hazard and paraffin oil has very small which is almost zero. Paraffin oil is miscible with isopropanol. Using higher concentration of paraffin oil in isopropanol mixture, light liquid paraffin oil reduces isopropanol effect by making it more stable compound [13].

1.2 Statement of the problem

Histopathology laboratory is an area where hazardous chemicals are in bulk with their solid, liquid, and vapor state with frequent and mandatory contact with these chemicals. Of these, Xylene is one of the most hazardous chemical which is known for its wide and routinely usage in tissue processing (clearing), staining (dewaxing) and cover slipping (mounting) purpose. All mentioned procedures are done in St. Paul’s hospital millennium medical college (SPHMMC) pathology laboratory using only xylene.

Laboratory professionals have high contamination risk with xylene chemical during their working times. Annually, an estimated 160 million new cases of work-related diseases occurred and an estimated 2 million people died worldwide, which is greater than the global annual number of deaths from malaria. A study done in Ethiopia by Semira R.*et.al*, (2019) showed that about 64% of pathology laboratory workers were affected by xylene contact [27, 70].

Xylene is a potential volatile occupational hazard for the histopathology staffs. Breathing its vapors in small amounts can cause headache, dizziness, anxiety, forgetfulness, inability to concentrate, drowsiness, abortion, and nausea. With more serious exposure, xylene can cause sleepiness, stumbling, irregular heartbeat, fainting, CNS damage or even death. Xylene vapors are mildly irritating to the skin, eyes, and lungs. It also causes increased blood urea, distal tube academia, decreased urinary clearance of endogenous creatinine, increased βglucuronidase, increased albumin, and RBC and WBC excretion. Xylene has fetotoxic effects like delayed ossification and behavioral effects, in the absence of maternal toxicity. Xylene inhaled by a woman can reach a developing fetus and can contaminate her breast milk. Therefore, Working areas with xylene vapor have high influence on laboratory personnel’s health, activity, productivity. This also in turn increases the tendency to make errors which are very disastrous [53, 54].

Human resources are the most significant resource in service provision. Poor working environment induces professionals turn over. Only 5-10% of workers in developing countries are estimated to have access to adequate occupational health services [18]. Job Motivation and satisfaction are highly integrated with safe working environment which in turn affects productivity. Xylene vapors produces discomfort on the pathology working personnel. Laboratory personnel are unable to work for long time in the laboratory which has in turn impact on existing turnaround (TAT) time. Processing less number of samples longer the TAT, hence it provokes early detection of diseases and influence medical intervention. Most hospitals histopathology laboratory has no hoods and well ventilation. Even its area of processing and staining is less than the national standard, 16sqm **[28, 70].**

Practical evaluations show that Minimum attention given for buildings of the pathology department. Observation of Four university tertiary hospital histopathology departments in Ethiopia including, Addis Ababa university-TASH, Mekele university-ACSH, Bahir Dar university (FHRH), and Hawasa university- HURH have very small and confined area of tissue processing, staining and mounting procedures. Even in these areas there is poor ventilation, some has no windows, difficult to move freely inside the laboratory causing inhalation, contact and splash of chemicals. In Ethiopia, about 76% of Pathology centers have below standard working rooms with poor ventilation [70].

Imported items like xylene are costly and induce shutdown times. Importing may sometimes be impossible in many instances, like in the case of COVID-19 when all international movements banned. Procedural, political, economic, social conditions and currency gaps also manifested frequent shortages of xylene and redundant shutdown of many pathology laboratories centers.

Unfortunately, currently, histopathology without xylene is unthinkable. There is Strong dependency of histopathology service on xylene presence which prone the service for interruption. On the other hand, the fluid and vapors of xylene cannot be prevented with glove and masks respectively which makes it very serious. The social, economic, health and psychological burden due to this chemical is vast.

Xylene can be absorbed into the blood via three routes including the lungs, the skin, and gastrointestinal. There is also no devices/mechanism/ used for exposure monitoring and degree of exposure measurement in all pathology laboratories in Ethiopia. Substitution is a major way for preventive mechanism. Majority of replacement trials were not successful. A study showed, of all trials, only very few were successful with many drawbacks and US histology laboratories replaced 41 % during tissue processing and 79% and 62% of them use machines for staining and cover slip mounting respectively. In Ethiopia there is no laboratory replaced for xylene during tissue processing [29 53, 54, 70].

In many studies, gaps were manifested with regard to method of study such as parameters incorporation, sample type selection and size determination. Empirical gap was also observed due to using unsatisfactory or erroneous way of determining 'adequacy for diagnosis' which is using 'scale method'. This method has both logical & practical discrepancy for histopathology slide adequacy determination.

Hence, substituting xylene with eco-friendly, non-toxic, relatively cheaper and easily available ismuch better than any other protective measures.

1.3 Significance of the study

Diagnosis with cancer staging, treatment selection, prognosis pursues, and complication prevention critically enforces availability of histopathology service. This service majorly uses boldly xylene which has effect on health, economic, productivity and safety issues. Tremendously hazardous nature of xylene with its far and wide health effect exacerbates the mess.

Utmost care is taken in planning and designing the study so that the result could be inferred to pathology laboratories which still are using xylene during tissue processing.

Finding Substitution of xylene with natural alternatives will give insight for federal ministry of health (FMOH), regional health bureaus (RHBs) university Hospitals, researchers, hospital management, and pathology department to see other alternatives for hazardous chemicals. It has also input for FMOH and Hospitals managements for reducing complaints and compensation payments raised by working personnel. It will also induce additional opportunity of acquiring new staffs.

The study will have implication for policy makers in shaping health policy framework in the country essential for launching appropriate and specific guideline and control strategies on xylene usage during tissue processing.

Reaching at substitutions for these imported reagents has also comparative advantage on the price aspect of reagents there by cost reduction for patients, institutions and the country.

Primarily, the working personnel will have a good working environment which can help him/her to spend most of the time at work. This improves productivity and reduces turnaround time. Finding substitution for xylene also enables health institutions efficient and effective use of the scarce pathology professionals with extended working hours and quality service delivery via induced healthy ergonomic. Thus the outcome of the research work is generally expected to benefit professionals, staffs working in histopathology laboratories in particular and the public in general.

Quality and safety officers of hospitals and other aromatic solvent users like industries can get new insight on substitution of hazardous chemicals. It also provides an insight for further study and can also be used as base for researchers who are interested in this area.

It is anticipated that the research will considerably be taken as base for further study by researchers' .It will also help academicians as a material for teaching students. It provides scientific knowledge for professionals through presentation, conference and publishing

Reducing or eliminating the use of this chemical through substitution has benefit in minimizing the pollution of the existing narrow laboratory rooms. Hence, evaluating the efficacy of PIE mixture substitution for xylene helps to pin point the gap and suggest solutions.

This finding is helpful for academicians, researchers and students to use it as an input to conduct further study and as teaching aid material. Researchers of pathology will find different methods of analysis for evaluating adequacy of slides for diagnosis.

Generally, this research will fill the gap in knowledge on substitution of the noxious chemical xylene in histopathology laboratory.

2 REVIEW OF LITERATURE

A study done in the department of Pathology at Sultan Qaboos University Hospital, Oman by *Alwahaibi NY et al; 2019,* evaluated ultra-Clear™ as both dewaxing and clearing agents. Thirteen different fresh surgical tissues were cut into two halves. One half processed using xylenes and the other half processed using ultra Clear. Total of 100 slides were cut from each method blocks. In this study six parameters including nuclear staining, cytoplasmic staining, cell morphology, clarity of staining, uniformity of staining and cost were evaluated. The result revealed that ultra-Clear™ showed only 76% (P = 0.016) adequacy. However, Ultra Clear™ is more expensive than xylene [30].

Another study was done in India by *WajidSermadi Z M et al, 2019,* on olive oil as xylene substitute .In this study, 60 soft tissue specimens from head and neck region were subjected to parallel processing in xylene and olive oil and stained with H&E for histologic evaluation. Gross changesof tissues, rigidity, cellular details, staining intensity and morphometric evaluations were done. The results showed no significant difference among methods except morphometric and rigidity. Recommending further studies, they concluded as olive oil can be substituted for xylene as a clearing agent [31].

Another study was done in the department of Oral Pathology and Microbiology, Mamata Dental College, Khammam, Telangana, India to compare the efficacy of xylene-free hematoxylin and eosin (H and E) sections with conventional H and E sections. Of the ninety, sixty blocks were processed with sesame oil and the rest with xylene. The study sample was divided into three groups. These Sixtysesame oil-processed blocks sections were stained with xylene-free H and E staining method. A 95% diluted lemon water and 1.7% dish washing solution were used as deparaffinizing agents. Slides were scored for diagnostic adequacy and staining quality. The result indicated 88.7%and 78% of 95% diluted lemon water and 1.7% dish washing solution were adequate for diagnosis respectively. Researchers concluded that tissues processed with sesame oil and stained using 1.7% DWS were found to be effective alternative to xylene [32].

Kerosene was tried in the Department of Oral Pathology and Microbiology, Vivekananda Dental College for Women, Tiruchengode, Tamil Nadu, and India for replacing xylene as a clearing agent during processing and staining. Thirty bits were collected processed using kerosene and stained in this study. The tissue blocks were subjected to sectioning and staining, and finally, they were observed under light microscope. Results revealed that Tissue samples that were processed and cleared with kerosene showed equal clearing and staining without any alterations of the tissue morphology and cellular details with that of xylene. However; kerosene is an aromatic hydrocarbon and not eco-friendly [33].

Bleached vegetable oil was also evaluated as a clearing and a dewaxing agent in department of Oral and Maxillofacial Pathology, DAPM RV Dental College and Hospital, Bengaluru, Karnataka, India. Twelve normal oral mucosal samples were cut into pairs forming two groups. One group was processed in xylene and others were processed in bleached vegetable (palm) oil. Tissue transparency, nuclear and cytoplasmic staining were assessed between the two groups. Good tissue transparency and serial sections were obtained. About 91.7% of sections treated with bleached palm oil revealed adequate nuclear and cytoplasmic staining [35].

Aparna. B et al, 2018, studied efficacy of dish wash solution, diluted lemon water, coconut oil and xylene as deparaffinizing agents for hematoxylin and eosin staining procedure. In the department Of Oral Pathology and Microbiology, Bapuji Dental College and Hospital, Davangere, Karnataka, India forty five blocks of various tissues were prepared .Five section slides prepared from each block and Stained with Hand E stain. Staining characteristics were evaluated. Chi square test was used for Statistical Analysis. The result showed adequacy of staining characteristics such as nuclear, cytoplasm, uniformity, clarity and crispiness of staining for diagnosis was greater with dish wash solution followed by diluted lemon water, coconut oil and xylene [36].

Another study was done in the department of Oral Pathology, S.D.M. College of Dental Sciences and Hospital, Dharwad, India .A total of sixty soft tissue specimens were processed in this study to replace xylene with coconut oil as a clearing agent and checked for gross and histological features. The result indicated no difference in cellular detail and staining quality. Significant shrinkage was noted in XY-S compared to that in CO-S. No difference was found in either of the sections when checked for cellular details and staining quality. Morphometrically, there was significant reduction in the mean cell area in XY-S compared to that in CO-S.They concluded as Coconut oil may be substituted for the highly hazardous xylene as a clearing agent [37].

Finding Bio friendly substitute for xylene emerged many trials. One of these was a research, *Premalatha BR et al*; 2103, on mineral oil as a deparaffinizing agent. Thirty paraffin-embedded tissue blocks were randomly retrieved from the archival collection of the Department of Oral Pathology, MS Ramaiah Dental College and Hospital, Bengaluru. Thirty sections were stained with conventional H&E method and 30 were stained using refined mineral oil. Two pathologists blindly analyzed using the parameters of uniformity, clarity and intensity of nuclear and cytoplasmic staining respectively. About 93.3% of sections in mineral oil were adequate for diagnosis (p-value 0.1500and they recommended refined mineral oil as a bio friendly and effective xylene substitute in deparaffinization of tissue sections **[38]**.

With one or more draw backs many researchers tried to find cheap and ecofriendly xylene substitutions. The costly cedar wood oil was also a target for researchers. An experimental study was done in India using thirty paraffin blocks of the routine biopsy specimen retrieved from the department archives. Sections were subjected to Essential oil (8% cedar wood oil) or xylene and stained with H and E stain. The stained sections assessed for nuclear and cytoplasmic details, clarity and uniformity of staining. Their result revealed that significant correlation was observed between cedar wood oil and xylene. They conclude that cedar wood oil can be an effective, eco-friendly and safe alternative to xylene as a clearing agent in the histopathological laboratory **[39]**.

A study was done by *K.Muddanna et al*; 2017, to know olive oil's possibility to substitute xylene as clearing agent.Thirty routine biopsy tissues of 1–2 cm were taken in Department of Oral and Maxillofacial Pathology, Kamineni Institute of Dental Sciences, Nalgonda, Telangana, India. Half processed with conventional method and the other half with olive oil as clearant instead of xylene. All the sections were stained with routine hematoxylin and eosin staining. On their result, Olive oil was found to be effective clearing agent compared to xylene **[40]**.

Saravanakumar P et al, 2019, stated that "pure groundnut oil" (GO) and "pure coconut oil" (CO) are efficient replacements for xylene with 45 delicate tissue samples. This study was done in Department of Oral and Maxillofacial Pathology and Microbiology, SRM Dental College, Chennai, Tamil Nadu, India. Cellular architecture and quality of staining were inspected. All the three groups showed similar results, and there was no difference identified when checked for cellular architecture and staining quality. They concludedthat both "pure CO" and "pure GO" has been shown to be an efficient replacement for "xylene" as a clearing medium, without jeopardizing on its histological features [41].

Another study was done in Australia to evaluate isopropanol as an alternative to xylene in the processing of tissue in anatomical pathology. Both normal and abnormal tissues were included. One half of the 20 specimens were processed with isopropanol and the rest with xylene. Haematoxylin and eosin (H&E), histochemical and immunohistochemical stains were performed and three anatomical pathologists blindly evaluated the quality of the slides produced. Recommending wider variety of tissues, HC, and IHC stains, the result showed Isopropanol's ability to replace xylene as clearing agent [42].

Liquid dish washing solution was assessed for its deparaffinizing capacity as a substitute for xylene in Hand E staining with and without xylene by *Ramulu* S.et al, 2012. In a study done in department of Oral Pathology, Navodaya Dental College, Raichur, Karnataka, India Fifty paraffin embedded blocks were included and two slides from each block prepared. The result showed about 90% of sections was adequate for diagnosis recommending LDWS usage as an alternative to xylene in Hand E staining procedure [43].

Clearing ability and bio-friendly nature of four different oils i.e., Carrot oil, Olive oil, Pine oil, Rose oil were compared with that of xylene. In this study, forty different formalin fixed tissue samples were taken. Total of 200 bits from 40 samples were subjected for clearing in 4 different oils on top of xylene as clearant. Cellular architecture and staining quality were assessed. Pine oil being superior, all oils showed ability to clear tissues similar to that of xylene. They recommended these oils as bio friendly and economical agents instead of xylene [44].

Gayle G.Andre and colleagues tried mixture of oils and paraffin for xylene substitution.in this study, liver, brain, and breast tissues were subjected to clearing and infiltration mixtures (CIMs) for xylene. Four CIMs prepared in 2; 1 ratio. Two parts of paraffin mixed with 1 part xylene / monosaturated, /unsaturated, / saturated oil. A fifth regimen was substitution of paraffin only for xylene. Their results showed about 97% were adequate for diagnosis. Hence, the experimental CIMs could be used as effective xylene substitutes for satisfactory processing of liver, brain, and breast tissue. These methods demonstrated minor difficulties in embedding, microtomy, or H&E staining procedures [45].

A study was done in Department of Allied Health Sciences, College of Medicine and Health

Sciences, Sultan Qaboos University, Muscat, Oman .Half of 230 blocks from 19 different tissues were prepared using xylene and UltraClear™ as a clearing agent. It is two times expensive than xylene. Including immunohistochemistry (IHC), and cost, quality of staining was evaluated. UltraClear™ processed sections showed 100%,81.7%) 67%, 60.9%, 52.2%, 63.5%, and 67% for IHC , easy to cut, nuclear staining, cytoplasmic staining, cell morphology, clarity of staining, and uniformity of staining, respectively. The researchers recommended the use of UltraClear™ solution as a routine clearing agent in histopathology laboratories. However, further studies are required [46].

3 OBJECTIVE

3.1 General Objective

The main objective of the study was evaluating efficacy mixture of paraffin oil, Isopropanol and ethanol as alternative for Xylene in surgical biopsy tissue processing by the conventional paraffin wax method.

3.2 Specific objectives

- To determine quality of processed tissues, blocks and stained slides of PIE mixture
- To compare quality of processed tissues, blocks and stained slides of PIE mixture with conventional xylene method
- To determine adequacy for diagnosis and turnaround time of PIE mixture in tissue processing
- To compare adequacy for diagnosis and turnaround time of PIE mixture with CXM in tissue processing
- To determine the substitution efficiency of PIE with xylene in histopathology processing.

4 MATERIAL AND METHODS

4.1 Study area and Setting

Figure 1: Study area image, St. Paul's hospital millennium medical college

The study was conducted at St. Paul's hospital millennium medical college, pathology department. It is located at Addis Ababa town Gulelle sub city. Addis Ababa is capital city of Ethiopia and residence of chair of Africa union. SPHMMC is one of the tertiary governmental hospitals in the country established in 1968E.C which has about 700 beds for admitted patients, including outpatient, inpatient, emergency, radiology, dermatology, surgery, trauma and other departments.

The hospital has pathology department which gives histopathology and cytopathology services. The patient registration log book of the department shows that in average 170 new cases visit histopathology unit of the department every week. Annually it gives nearly for 10,000 surgical samples in its histopathology unit. All units and departments of SPHMMC and other health institutions in the country send surgical biopsy samples to the department for diagnosis.

The department is also engaged in researches related with different diseases and providing postgraduate program in residency. It is equipped with different types of instruments such as Microtome, Tissue Embedding Console, Cryostat, Tissue Processor, Incubator, Refrigerators and Safety-hood. Currently the pathology department has eleven senior pathologists, fourteen pathology residents, three senior histologists, eight medical laboratory technologists, one informationtechnology technician, one secretory, and three receptionists [71].

4.2 Study Design

A comparative cross-sectional study was conducted to assess the clearing quality ofPIE mixture.

4.3 Data collection tools and data quality assurance

Checklists were prepared to assess the quality of blocks and slides for sectioning and microscopic evaluation by histotechnologists and pathologists respectively.

4.4 Study period

The study was conducted in February 2019 up to December 2020 GC at St. Paul's hospital millennium medical college.

4.5 Source of sample

The source of sample for the study was all biopsy samples, of St. Paul's hospital millennium medical College.

4.6 Study sample

It was biopsy samples processed during the study period excluding Small samples with no extra tissue for second cassette.

4.7 Eligibility

4.7.1 Inclusion Criteria

All biopsy samples which were fit for grossing and whose bits were going to be taken during the study period.

4.7.2 Exclusion Criteria

Biopsy samples with insufficient formalin fixative or with other fixatives were excluded. Also samples which were received during the study period but not handled as per the standard operating procedures (SOP) of the unit. Again Small samples with no extra tissue for second cassette were excluded.

4.8 Sample size Determination

New tissue processing evaluations recommend focusing on involving many tissue types and use of autopsy and surgical specimens ensuring equal probability. The national committee for clinical laboratory standard (NCCLS), 2011 guideline recommends a minimum of 40 samples for method comparison. It recommends large sample size (100 to 200) to assess whether new method is similar to that of the comparative method [37]. Keeping in touch with these points a total of 112tissue biopsy samples were selected by convenient sampling method.

4.9 Sampling method

Convenient sampling technique was used based on the type of tissue. From each type of tissue one cassette was selected. For tissue types which were received more than one per day only the first were sampled. Total of 112 biopsies were selected based on the type of tissue.

4.10 Variables

4.10.1 Dependent variable

- Block quality
- Microtomy
- Transparency
- Nuclear staining
- Cytoplasmic staining
- Clarity of staining
- Uniformity of staining
- Crispness of staining

4.10.2 Independent variable

- Xylene free processing /PIE/ method
- Conventional processing/CXM/ method

4.11 Study procedure and data collection

4.11.1 Materials and Reagents

Materials include: *Leedo* Tissue processor, cassettes, surgical biopsy samples, *SLEE* auto strainer and microtome, processing reagents, staining reagents, mounting reagents, microscope, camera, ruler, sample containers, stationeries, inks, and guans. (Reagents used: Isopropanol-CARLO ERBA, Italy, expire date 2023. Light liquid paraffin oil, panreac Aplichem, Italy expire date 2022. Ethanol, Alpha chemika, Italy, expire date 2025)

The PIE tissue processing method used paraffin oil, isopropanol and absolute ethanol for clearing tissue samples during tissue processing as an alternate substitute for conventional xylene .The PIE mixture was prepared by increasing the concentration of paraffin oil while decreasing that of ethanol and isopropanol to the extent of totally using paraffin oil only. Paraffin oil, isopropanol and ethanol were mixed in 2:1:3 and 3:1:2 in the first two beakers respectively and 100% light liquid paraffin oil. For the conventional method only xylene was used.

The most significant variables in tissue processing are temperature, characteristics and concentrations of the reagents and properties of the tissue. Processing reagents to specimens' volume ratio was maintained at 1:20. Accordingly these variables were managed for effective tissue processing in both methods [67, 68].

Mayer's hematoxylin was used for both CXM and PIE processing 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 additionof 0.5 ml acetic acid per liter.

4.11.2 Tissue samples and collection procedure

Data collected every day on the type, nature, size, type of tissue from the log book. The data were collected by principal investigator (PI). Purposefully the number of biopsy samples determined. Hence, the type of tissue was equal to the number of cassettes. Total of 112 biopsy samples were studied and purposive sampling technique utilized to arrive at the target. Procedure wise, tissues were selected based on type. For each type of tissue there were two similar bits cassettes numbered sequentially and alphabetically. To achieve for complete randomization for selecting processing method, coin was tossed assigning head and tails for each type.

4.11.3 Histological processing and staining procedure

One hundred twelve different formalin fixed tissue samples were procured from the pathology department of SPHMMC, Addis Ababa. Biopsy specimens were received in the laboratory in containers filled with 10% formalin fully covering the tissue sample.

Prefixed tissue slices should not be thicker than 1.5 mm. Hence, small biopsy specimens require no trimming while large specimens are trimmed on a dissecting board specifically developed to prepare uniform slices of desired thickness.

From Each sample two similar and equal bits were taken and put in their respective cassettes. These cassettes were labeled consecutive numbers accompanied by letters for identification, one with alphabet A and the other with B. Each pair of cassettes was put in a basket. Total of 112 cassettes were kept in each basket, and then one pair was sent for conventional xylene tissue processing, another for PIE tissue processing.

Depending on the proportion of each component, the mixture becomes absolute transparent at different temperatures. It happens at 45 ^{o}c and 44 ^{o}c in a mixture of One –sixth and one -third Paraffin oil respectively [48]. Ethanol is dehydrating agent. Isopropanol is both dehydrant and lipid extractor. Liquid paraffin oil is a clearing agent. Hence it is understandable that the proportion of ethanol has to decrease and that of paraffin oil increasing; keeping isopropanol constant proportion. Isopropanol used as an intermediate in many aspects. It is miscible with water and ethanol. Its oil dissolving ability is helpful for mixture formation. The viscosity of isopropanol is inversely related with temperature. When the concentration of paraffin oil increases and that of ethanol decreases leads to a decrease in temperature required to get absolutely transparent mixture.

In the new method, two changes of PIE mixture and two changes of pure light liquid paraffin oil is used as a clearing agent instead of three changes of xylene in conventional method. Conventional automated overnight tissue processing method using xylene and substitute mixture were employed. Following paraffin impregnation, the processed tissues were embedded, trimmed, sectioned at 4 micron thickness, floated, stained with H and E stain and mounted. One paraffin section of four micron thickness was cut from each of the one hundred twelve paired paraffin blocks of CXM and PIE method processed tissue specimens.

Table 1: Tissue processing procedures of PIE and CXM using *Leedo* linear tissue processor

Station	Step	Chemicals and time used					
		PIE method			Conventional xylene method		
		Chemicals	T°	time	Chemicals	T°	time
1	Fixation	Formalin	40°c	30'	Formalin	40 °c	0:30
2	Fixation	Formalin	40°c	30'	Formalin	40 °c	0:30
3	Dehydration	40% ethanol alcohol	40°c	1:00 hr	70% ethanol alcohol	40 °c	1:00 hr
4	Dehydration	80% ethanol alcohol	40°c	1;00 hr	80% ethanol alcohol	40 °c	1:30
5	Dehydration	96% ethanol alcohol	40°c	1:00 hr	96% ethanol alcohol	40 °c	1:30 hr
6	Dehydration	Absolute ethanol alcohol	40°c	1:00 hr	Absolute ethanolalcohol	40 °c	1:30 hr
7	Dehydration	Absolute ethanol alcohol	40°c	1:00 hr	Absolute ethanol alcohol	40 °c	1:30 hr
8	Dehydration	Absolute ethanol alcohol	40°c	1:00 hr	Absolute ethanolalcohol	40 °c	1:30 hr
9	Dehydration& / clearing	Liquid paraffin, Isopropanol and Ethanol mixture	40°c	1:00 hr	Xylene	40 °c	1:30 hr
10	Dehydration& / clearing	Liquid paraffin, Isopropanol and Ethanol mixture	40 °c	1:00 hr.	Xylene	40 °c	1:30 hr
11	Clearing	Liquid Paraffin oil	65°c	1:00 hrs.	xylene	40°c	1:00 hr
12	Clearing	Liquid Paraffin oil	65°c	1:00 hrs.	Paraffin wax	65°c	1:00 hr
13	Infiltration	Paraffin wax	65°c	1:30 hrs.	Paraffin wax	65°c	1:00 hr
14	Infiltration	Paraffin wax	65°c	1:30 hrs.	Paraffin	65°c	1:00 hr
	Total			**14:00**			**16:30 hrs.**

Staining was done by using *SLEE* autostainer machine with manual mounting. All the rest were similar to the conventional methods done as per standard operating procedure manual of the histopathology unit.

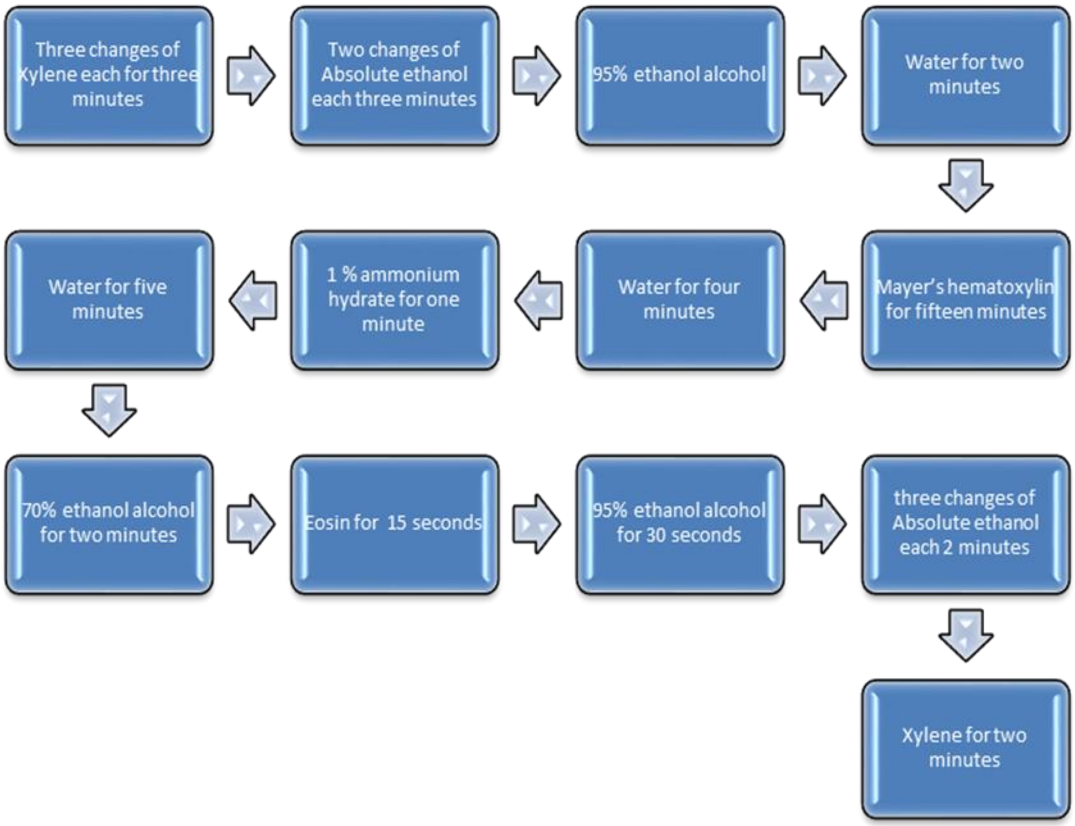


Figure 2: H & E staining procedure using SLEE autostainer

4.11.4 Assessment of processed tissue, blocks and stained slides

The resulting blocks were only numbered with no indication of the processing method. Each final block was evaluated blindly by the histologists who tabulated the cutting characteristics of each block. On the other side, each produced slide stained with routine staining was blindly evaluated by pathologists. For each pair, pathologists and histologists selectedone slide over the other.

Tissue transparency, shrinkage and cutting characteristics of tissues after processing were evaluated. Without prior information about the method employed for processing, three histologists evaluated tissue transparency, and simplicity to be sectioned. They also evaluated the storage quality of the block after a week for tissue shrinkage.

Tissue sections obtained by the two methods were stained simultaneously with Mayer's H&E.

The slides were independently evaluated by five pathologists for quality of staining and nuclear – cytoplasmic detail .The TAT of the two methods was also evaluated by the principal investigator (PI). H and E stained sections were graded based on the parameters of nuclear (adequate, score=1, in adequate, score=0), cytoplasmic staining (adequate, score=1, inadequate=score 0) and uniformity of staining (present=score 1, absent, score=0).The score for each slide were added. Maximum score would be five and minimum be zero. A score of $\leq$2 were graded as inadequate for diagnosis, and the slides with score, 3-5 were assigned as adequate for diagnosis [49]. Pathologists has recorded adequacy of slides for diagnosis.

4.11.1 Pilot study

Seven Pilot studies were performed with different tissue types on 10 samples at SPHMMC histopathology unit before the actual research. In the first two pilot studies heavy liquid paraffin oil at room temperature were tried and there happened a major discrepancy on tissue microtomy. To alleviate this problem, in the next pilot studies, light liquid paraffin oil was used which was mixed with different proportions of isopropanol and ethanol. Initially the temperature of the mixture was maintained at room temperature which showed still some discrepancy in the transparency and homogeneity of the mixture. Finally in the next three pilot studies light liquid paraffin oil with the same mixtures of isopropanol and ethanol were maintained at various temperatures of 40 ^{0}c, 50 ^{0}c and 65^{o}c.

From the pilot studies there was an experience that the temperature had to be adjusted at 65^{0}c and the mixture of paraffin oil, isopropanol and ethanol be maintained in 3:2:1 And 1:2:3 proportions followed by the pure light liquid paraffin oil. Time was tried to be adjusted in these pilot studies and it was found convenient that the time should be at 1hr in all four clearing solutions. Based on the pilot study findings, the actual research was done.

4.12 Data quality assurance

4.12.1 Pre analytical

All biopsy samples were checked for adequacy of fixation and fixative with free of decomposition. They were processed with the same tissue processor at once using “Leedo” open type linear tissue processing machine. All tissue processing reagents, fixation, dehydration, clearing, infiltration, staining, and mounting media were new or freshly prepared and checked for their effectiveness. Tissue floatation bath waters were kept free from floaters. All processing and staining reagents were prepared as per the standard operating procedure of the laboratory checking the expiration date for every powder, reagent or solutions used. For both methods; embedding, sectioning, mounting and staining were done by the same machines

For data quality, orientations were given for accession staffs, histologists and pathologist. Pretest was done to ensure acceptability, comprehensiveness, and understandability by the participants. Regular supervision, spot checking and reviewing the completed checklist carried out daily by the principal investigator.

4.12.2 Analytical

Based on the standard operating procedure of the histopathology laboratory; standard processing, embedding, sectioning, and staining procedures were followed. Both the new method and the conventional tissue processing methods were performed by the principal investigator (PI) .All the histologists and pathologists were blinded and were experienced from three up to eight years.

4.12.3 Post analytical

Checking and entering data on daily bases performed. All the entered data checked before final analysis. All the data were registered and entered to SPSS version 26 by the PI.

4.13 Method of analysis

A structural evaluation check list was prepared for evaluators and filled by them for qualitative data analysis of blocks and slides. Data on tissue transparency block simplicity to be sectioned, storage characteristics, nuclear and cytoplasmic staining characteristics based on uniformity, clarity and integrity (crispy) were recorded.

After data collection from check list of evaluators were processed and analyzed with the outline. In the first phase of data processing, I edited the collected raw data to detect errors, omissions, and unnecessary data and for possible correction. Then it was coded, classified and tabulated so that it can be controlled to analyze.

The statistical package for social science (SPSS) Version 26 was used in processing the data obtained through check list. McNemar's test was performed to show the equivalence between the matched paired blocks and stained slides processed via PIE and CXM methods, $p<0.05$ considered as not statistically significant. Chi -Square was also used to examine the differences between categorical variables between the two Method, $p<0.05$ considered as statistically significant.

Total evaluations of adequacy for diagnosis were done using Score method, evaluators' record, and Leica methods. The Leica, system of new method evaluation was also used. LEICA' system for 'new method evaluation'': uses the percentage average of macroscopic and microscopic: ≤ 50% - poor, 70%-80% possible to use but not substituent, ≥ 80% are excellent /substitute/. Score method interpreted as adequate if the sum of microscopic evaluations is greater or equal to three other wise it is inadequate. Pathologists' direct microscopic determination on the adequacy or inadequacy of the slides was also analyzed.

Quantative explanations like percentages were made from the data to give meaning to them as well as their implication. From these, appropriate conclusions and recommendations were made on the findings of the research.

4.14. Ethical considerations

Approval for the ethical clearance obtained from Addis Ababa University, college of health science, school of medicine, pathology department. Since data collection conducted from biopsy samples of histopathology unit and as the study did not directly involved patients in any way, there was no direct risk to study subjects from participating in the study. Therefore, informed consent was not sought from the study subjects. But I made sure that confidentiality of the information assured in such a way that no disclosure of any name of the patient, pathologist or histotechnologists in relation to the finding made. A letter was also forwarded from AAU pathology department to SPHMMC pathology department for collaboration and all possible support in the study.

4.15. Result dissemination

The result of this finding will be disseminated to Addis Ababa University, pathology department, hospital safety and quality assurance office, Mekele University, SPHMMC, FMOH, RHBs, University hospitals with histopathology service, EFMHACA. The result will be presented on different seminars, meetings and workshops. Manuscript will be submitted for publication on local or international peer reviewed journal.

5. RESULTS

A total of one hundred twelve biopsied tissues were grossed and cut into two equal parts. For each biopsy specimen two cassettes of tissues were taken. A total of 224(112 paired) cassettes processed using conventional and PIE method .One part was processed by conventional method and the rest by PIE rapid method. Hence, each method processed 112 cassettes. The blocks and slides obtained after processing were circulated among eight evaluators (three histologists and five pathologists) for evaluation.

A block was prepared from each cassette. These blocks evaluated for their storage and cutting (sectioning) quality. All the blocks were evaluated by three histologists independently. All histologists used a Checklist that contains five main parameters. From the five parameters, those slides that only fulfilled the three –forth or more parameters were considered adequate for diagnosis.

Again, from each block a slide was prepared and assessed for their staining quality by pathologists. All the slides were evaluated by five pathologists independently. All pathologists used a checklist that contains five parameters. From the five parameters, those slides that only fulfilled the three-fourth or more parameters were considered adequate for diagnosis.

Finally, Assessment result of blocks and stained slides quality were used determine the efficacy to substitute conventional method for PIE method.

The result of this experimental data has been presented starting from a background of samples processed up to turnaround time evaluation. It was structured in the following way.

1. Back ground sample distribution
2. Block and its sectioning quality
3. Cytoplasmic and nuclear staining characteristics
4. Uniformity and clarity of sections
5. Crispness characteristics of sections
6. Substitution capability of PIE mixture
7. Turnaround time PIE mixture

5.14.Samples distribution

The study was conducted on a total of 112 biopsy specimens. Different kinds of tissue samples were included: Colon, Brain, Liver, Breast ,Thyroid, Kidney, Breast ,Uterus, Stomach ,Skin, Bladder ,Prostate ,Cervix ,Gall Bladder ,Fatty tissue ,Endoscopic, Lymph Nodes, Bone marrow, ovarian ,cystic masses, Adrenal, neck masses ,Fallopian tubes, pancreas, and Lung tissues.

5.15.Block quality and Sectioning quality

Histologists assessed block quality, easy of sectioning/cutting/, mounting, and physical quality of sections Easiness of block cutting was assessed through judgment of ribbon formation, cohesiveness and uniformity of the sections parameters. Mounting was assessed by checking absence of Sweating, Cohesiveness and Flattening of the sections during tissue floatation.

5.15.1. Cutting

Histologists (I and III) stated that 94.9% of CXM blocks were easy to cut. They also recorded that 96.1% and 93.8% of PIE were easy, respectively. Histologist II mentioned that 93.5% and 93.8%of the CXM and PIE method were suitable for sectioning respectively. The result showed no significant difference among the two methods ($p>0.005$).

All together, the three histologists registered that 94.4% of the CXM and 94.5% of the PIE were adequate for thin cutting. Of the three parameters, ribbon formation, cohesiveness, and uniformity, all histologists favored Cohesiveness and uniformity for PIE and ribbon formation for the CXM. The result manifested no significant difference among the two methods ($p>0.005$, $p=0.967$). (Table 2)

Table 2: Blocks cutting/microtomy/ comparison PIE and CXM processing methods

Histologist	Tissue processing methods											
	Conventional xylene (CX) (n=112)						PIE(P) (n=112)					
	Ribbon without compression		cohesiveness		uniformity		Ribbon without compression		cohesiveness		Uniformity	
	Present	absent	Present	absent	Present	absent	Present	Absent	Present	absent	Present	Absent
I.	99	13	110	02	110	02	102	10	110	02	111	01
II.	96	16	109	03	109	03	96	16	109	03	110	02
III.	101	11	109	03	109	03	97	15	108	04	110	02
Total	**296**	**40**	**328**	**08**	**328**	**08**	**295**	**41**	**327**	**09**	**331**	**05**
Percentage	**88**	**12**	**97.6**	**2.3**	**97.6**	**2.3**	**81.9**	**12.2**	**97.3**	**2.5**	**98.6**	**1.4**
Over all	**952/1008=94.4%**						**953/1008=94.5%**					

5.15.2. Mounting

Three histologists registered that 98.7% of the CXM and 98.2% of the PIE were adequate for mounting. All histologists declared absence of sweat during floatation. There is significant difference between the two processing methods in terms of tissue mounting during floatation p=1.000).

Rating CXM, 98% was identified to have appropriate floatation by histologists (I and III); while 98% and 99% of PIE floated perfectly by Histologist I and III respectively. The result briefed no significant difference among the two methods (p=1.000).

During floatation, 100 % of CXM and PIE had no sweat on tissue floatation bath. Section Cohesiveness in the water bath observed in 97.9% and 94.3% of CXM and PIE respectively.(Table 3).The result shows there was no significant difference among the two methods ($p>0.05$).

Table 3: Mounting parameters (sweating, cohesiveness, and flattening) for CXM & PIE sections

Histologist	Tissue processing methods											
	Conventional xylene (CX) (n=112)						PIE(P) (n=112)					
	Sweating		Cohesiveness		Flattening		Sweating		Cohesiveness		Flattening	
	Present	absent	Present	absent	Present	absent	Present	absent	Present	Absent	Present	absent
I	0	112	109	03	111	02	0	112	109	03	108	04
II	0	112	108	04	111	01	0	112	107	05	109	03
III	0	112	109	03	110	02	0	112	111	01	111	01
Total	**0**	**336**	**326**	**10**	**332**	**05**	**0**	**336**	**327**	**09**	**328**	**08**
Percentage	**0**	**100**	**97.9**	**2.1**	**98.8**	**1.2**	**0**	**100**	**97.3**	**2.7**	**97.6**	**2.4**
Over all	**995/1008=98.7%**						**991/1008=98.2%**					

5.15.3. Block quality on stability during storage

Histologists assessed block Shrinkage and opacity as parameters to report the block stability. Block storage quality judgments done a week after blocks sectioned. From the study, three histologists reported that shrinkage was exhibited in less than 1% of those prepared by CXM while PIE blocks revealed no specimen shrinkage. Less than 1 % of opacity was found in PIE method. Block quality during storage proclaimed PIE methods is esteemed in preventing specimen shrinkage over conventional method. The result announced no significant difference among the two methods in terms of stability of block during storage ($p>0.05$, $p=1.000$).

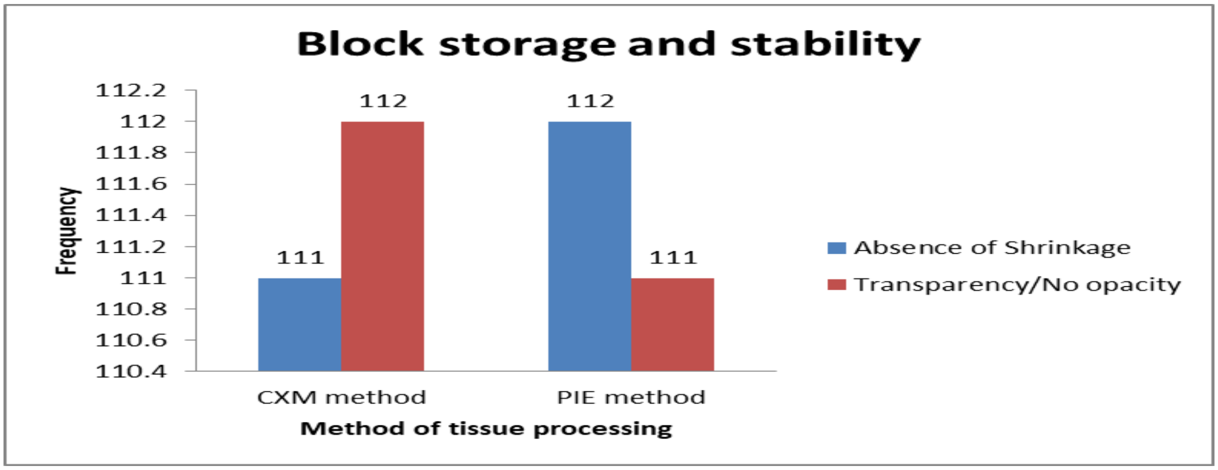


Figure 3: Comparison of processing methods for block quality and stability during storage (shrinkage & opacity)

5.16.Physical quality of section excluding stain quality

In this study, there was examination of the physical quality of the sections. It was done microscopically by gauging presence or absence of section disruption (holes and tears in sections), small or large cracking, and uniformity of thin section and flattening sections on the slide.

All sections prepared by the three histologists adhered perfectly to the slide with the exception of one slide (0.3%) in PIE method. Microscopically around 2.7 % manifested variation in section thickness in case of PIE method. About 97.9 % of both CXM and PIE method sections showed uniformity and no disruption which is good physical quality. All histologists reported equality of the two methods in terms of physical quality of sections (98.3%, p=1.000). This result announced no statistical significant difference among the two methods indicating equivalency of the two methods in terms of producing good physical quality sections.

Table 4: Physical quality of sections slides in PIE and CXM processed tissues.

Histologist	Tissue processing methods											
	Conventional xylene (CX) (n=112)						PIE(P) (n=112)					
	Adhesion		Cracking (disruption)		Section Thickness uniformity		Adhesion		Cracking (disruption)		Section Thickness uniformity	
	Present	absent	Present	absent	Present	absent	Present	absent	Present	absent	Present	absent
I.	112	0	3	109	107	05	112	0	04	108	108	04
II.	112	0	2	110	110	02	111	1	01	111	110	02
III.	112	0	2	110	109	03	112	0	02	110	109	03
Total	**336**	**0**	**7**	**329**	**326**	**10**	**335**	**1**	**07**	**329**	**327**	**09**
Percentage	**100**	**0**	**2.1**	**97.9**	**97.1**	**2.9**	**99.7**	**0.3**	**2.1**	**97.9**	**97.3**	**2.7**
Remark	**991/1008=98.3**						**991/1008=98.3**					

5.17.Preference

103(91.9%) of CXM and 101 (90.1%) of PIE were easy to section by Histologist I. Histologist I notified disagreements in 20 (8.9%) blocks. Eleven favored CXM and nine for PIE. This result shows no statistical difference among methods in terms of preferences indicating equivalency. (P> 0.05)

There were 104 (92.8%) blocks of CXM and 107 (95.5%) of PIE were easy to cut for Histologist II. Histologist II has recorded discrepancy of 13(5.8%) blocks .Eight favored PIE while five for CXM slides. No statistical significant difference observed among methods.(P>0.05).

Histologist III recorded that 108(96.4%) and 106(94.6%) 0f CXM and PIE blocks were easy to cut. There were 10 unpaired blocks mismatched. Six esteemed CXM and 4 paired PIE method. This result shows no statistical no difference among methods indicating equivalency.(P>0.05)

Assessment of the three histologists indicated that 93.4% and 93.7% blocks were able to produce good sections with PIE and CXM methods respectively; the result showed no statistical difference among methods in terms of preference(P=1.000). From their report, Histologists II preferred the PIE method while histologists (I and III) favored for conventional method.

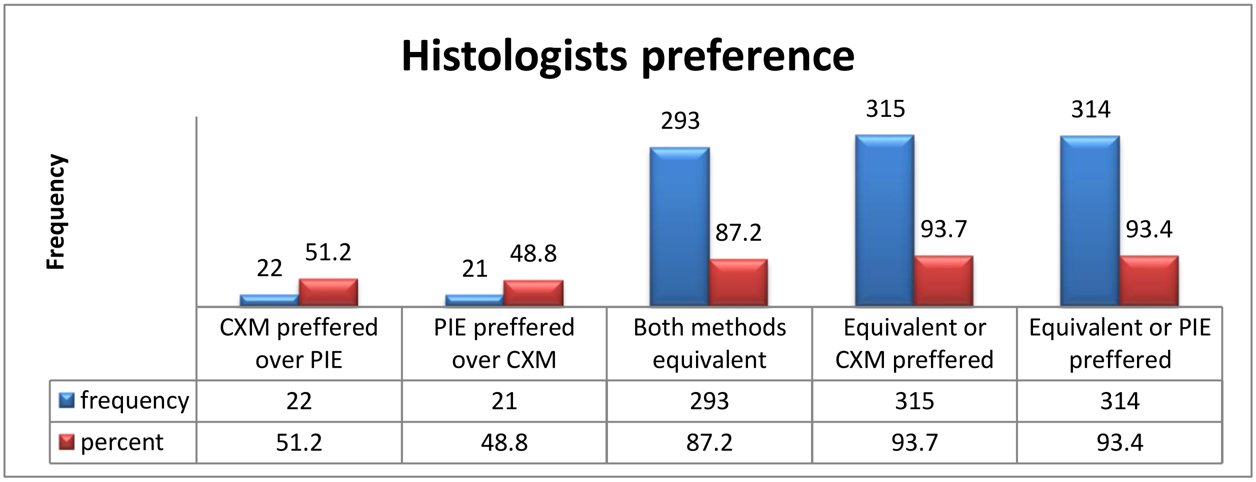


Figure 4: Method preference of histologists over the CXM and PIE processing methods

5.18.Microscopic evaluation

5.18.1. Nuclear and cytoplasmic staining

5.18.2. Nuclear staining

The results showed that of the 112 paired sections studied, 95.7% of CXM slides showed adequate nuclear staining as compared with 95% of PIE [Table 5]. The difference was not statistically significant (p=0.601) suggesting that there was no difference in the two processing methods in terms of producing adequate nuclear staining.

Two pathologists, pathologist B and D, reported 106(94.6 %) of CXM slides were good for nuclear staining .Respectively, 107(95.5 %) and 105(93.7%) of PIE slides had good nuclear stain by pathologist B and D. Nuclear staining was reported competent in 108(96.4%) with CXM and 104(92.8%) with PIE by pathologist E.

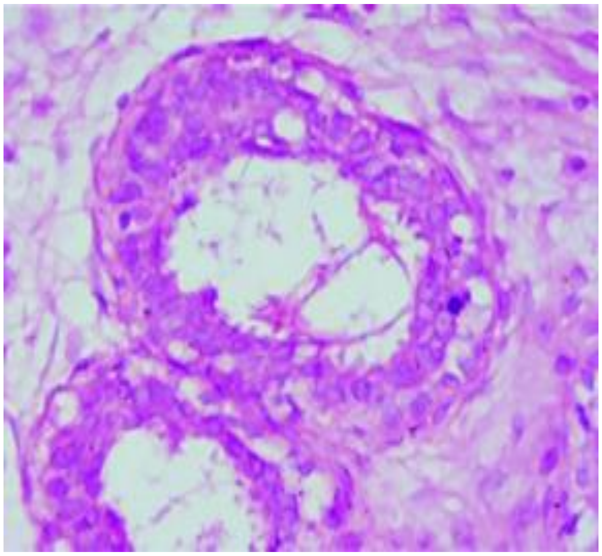
Pathologist A indicated that 97.3% of both CXM and PIE slides were adequate for nuclear staining. Similarly Pathologist C assessed both CXM and PIE method were equivalent manifesting equal 107(95.5%) adequacy of nuclear stain.

Adequacy of nuclear stain was equal by both pathologists (B and D) with CXM but in their PIE method pathologist B reported 109 favoring PIE while pathologist D affirmed 105 adequacy favoring CXM.

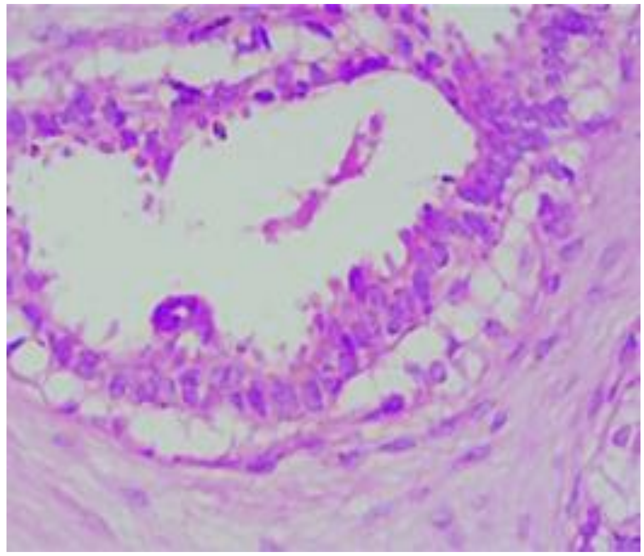
Pathologist E stated that 96.4% of CXM and 92.8% of PIE had good nuclear stain. Among the three pathologists (B, D and E) who evaluated112 paired slides, disagreement reported only in six slides. No disagreement registered by pathologists A and C.

Table 5: Adequacy of nuclear staining in CXM and PIE processed tissues

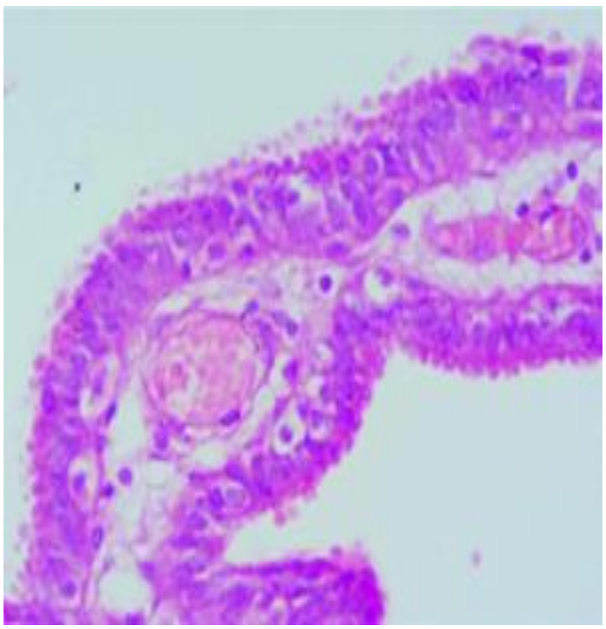
Pathologist	**Conventional (n=112)**			**PIE(n=112)**		**Significance**
		Number	**Percent**	**Number**	**Percent**	**p-value**
A	Adequate	109	97.3	109	97.3	(P=1.000)
	Inadequate	03	2.7	03	2.7	
B	Adequate	106	94.6	107	95.5	(P=0.775)
	Inadequate	06	5.4	05	4.5	
C	Adequate	107	95.5	107	95.5	(P=1.000)
	Inadequate	05	4.5	05	4.5	
D	Adequate	106	94.6	105	93.7	(P=0.789)
	Inadequate	06	5.4	07	6.3	
E	Adequate	108	96.4	104	92.8	(P=0.270)
	Inadequate	04	3.6	08	7.2	
Over all Total	**Adequate**	**536**	**95.7**	**532**	**95**	**(P =0.601)**
	Inadequate	**14**	**4.3**	**18**	**5**	



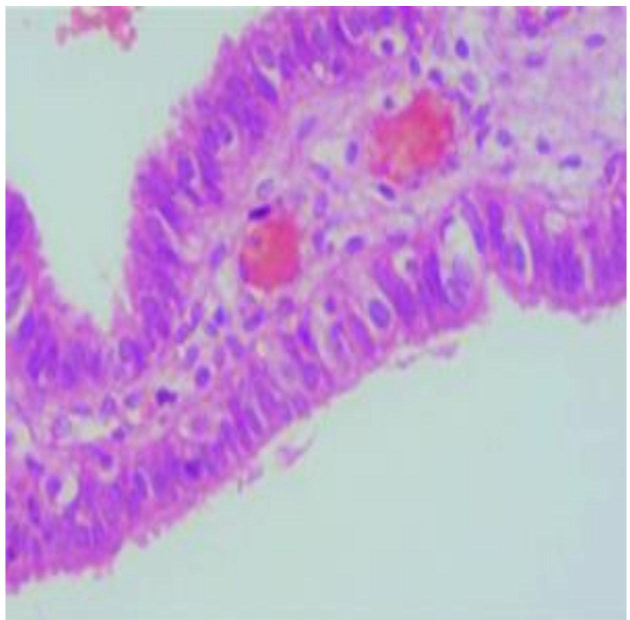
Breast 4OX-PIE



Breast 40x-CXM



Fallopian tube 40X-PIE



Fallopian tube 40X-CXM

Figure 5: Photomicrography showing comparison of nuclear stain of CXM & PIE processed tissues and H & E stained sections (40X) & (40X) - PIE: (40X) & (40X) - CXM

5.18.3. Cytoplasmic staining

Adequate cytoplasmic staining was accounted in 96.1% of CXM sections as compared to 96.4% of PIE sections and statistically there was no significant difference in the two processing methods in producing adequate cytoplasm staining. (P>0.05, p=0.772)

From all one hundred twelve PIE processed and Hand E stained slides that were used to assess the cytoplasmic staining 105(93.7%), 107(95.5 %), and109 (97.3 %) were adequate by pathologists A, B, and D respectively. Section slides produced via CXM showed adequacy in 107(95.5 %) by Pathologist A, B and D.

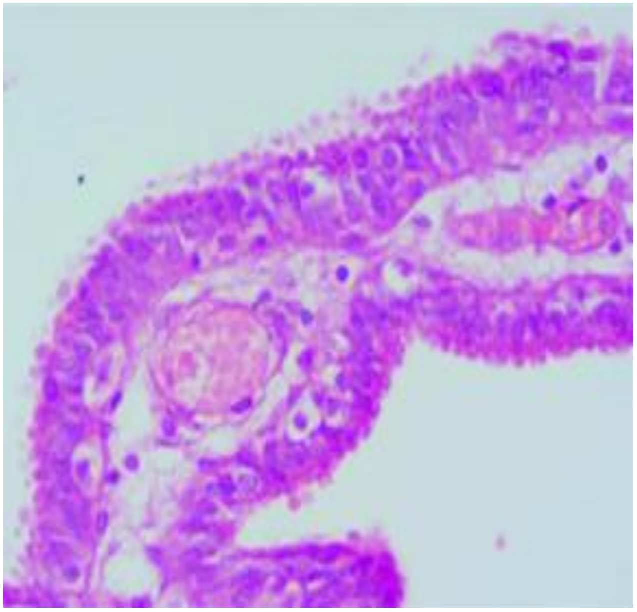
There were 6 unpaired mismatches by pathologists A and D. Four favored for PIE and the rest two for CXM. Pathologist B reported no difference among the methods. The result showed no significant difference among the two methods (Table 6).

Pathologist C reported 109(97.3 %) of PIE and 106(94.6 %) of CXM processed tissues had adequate cytoplasmic staining. Five unpaired slides showed difference as per pathologist C, four were in favors PIE method while one for CXM. No statistical significant difference observed among the two methods (p>0.05) (Table 6).

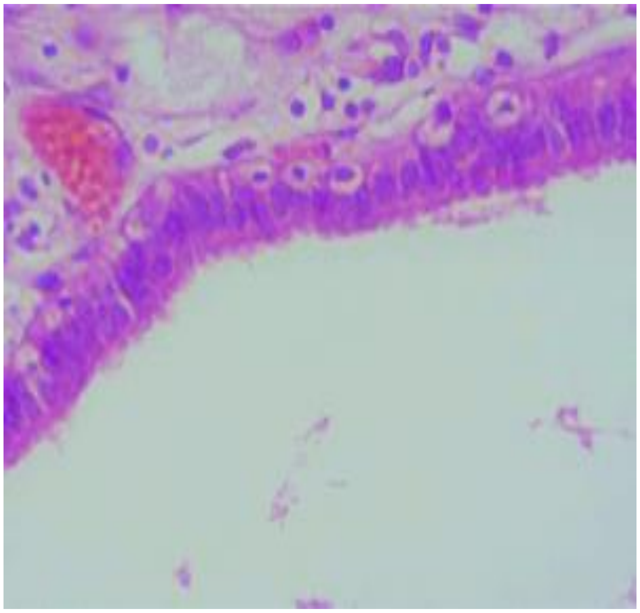
As compared to 110(98.2 %) adequately cytoplasmic stained sections of PIE, Pathologist E reported 111(99.1%) of CXM processed tissues had adequate cytoplasmic staining. only one unpaired slides showed discrepancy that favors for xylene method. The result indicated no statistical significant difference among the two methods (p>0.05) (Table 6).

Table 6: Adequacy of cytoplasmic staining in CXM and PIE processed tissues

Pathologist	Conventional (n=112)			PIE(n=112)		Significance
		Number	Percent	Number	Percent	p-value
A	Adequate	107	95.5	105	93.7	(P=0.581)
	Inadequate	05	4.5	07	6.3	
B	Adequate	107	95.5	107	95.5	(P=1.000)
	Inadequate	05	4.5	05	4.5	
C	Adequate	106	94.6	109	97.3	(P=0.354)
	Inadequate	06	5.4	03	2.7	
D	Adequate	107	95.5	109	97.3	(P=0.518)
	Inadequate	05	4.5	03	2.7	
E	Adequate	111	99.1	110	98.2	(P=0.561)
	Inadequate	01	0.9	02	1.8	
Over all Total	**Adequate**	**538**	**96.1**	**540**	**96.4**	**(P=0.772)**
	Inadequate	**22**	**3.9**	**20**	**3.6**	

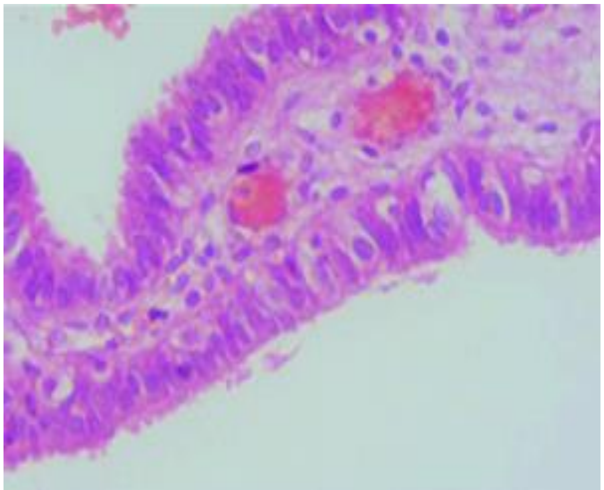


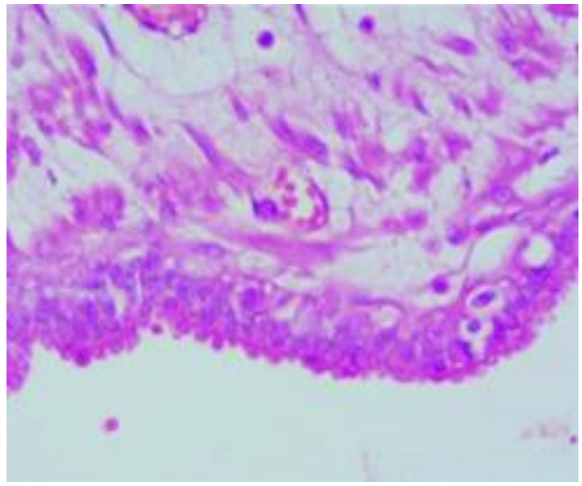
A



B

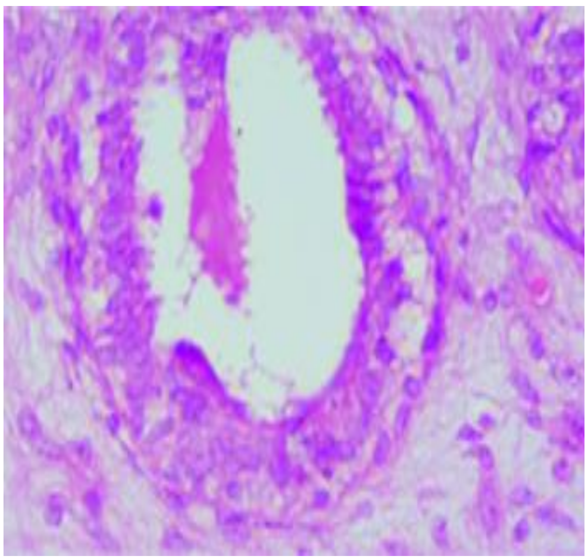
Fallopian tube -PIE -40X (A &B)

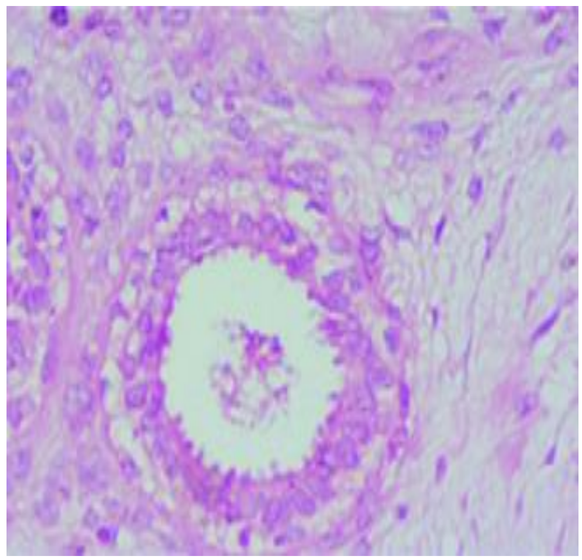




C D

Fallopian tube -CXM -40X(C &D)





E F

Breast 40X-PIE Breast 40x-CXM

Figure 6: Photomicrography showing cytoplasm stain comparison of CXM & PIE processed tissues stained with Hand E stain:(A -40X, B -40X)-PIE: (C -40X, D -40X)-CXM: (E -40X-PIE) & (F -40X-CXM)

5.19.Uniformity and clarity characteristics of sections

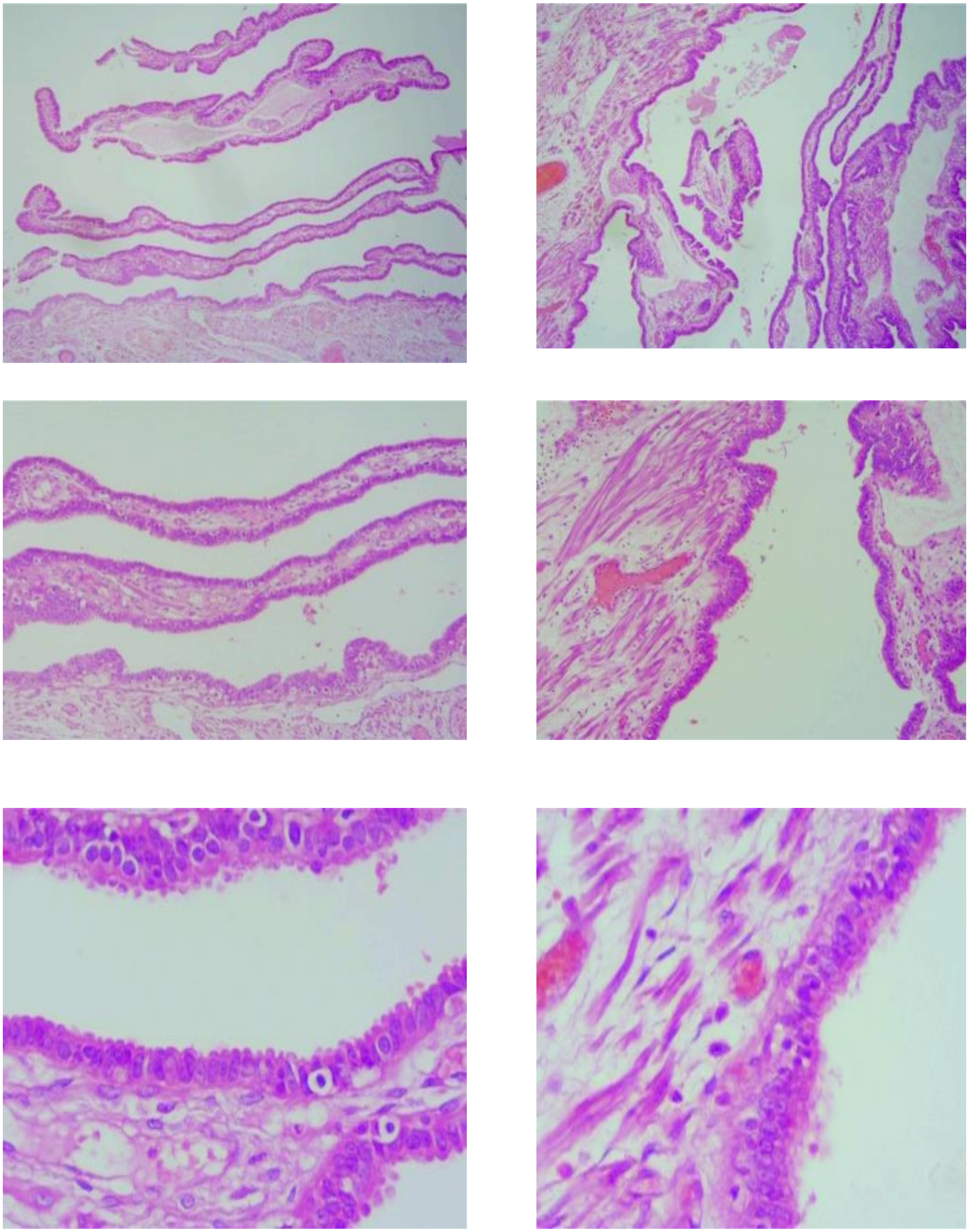
5.19.1. Uniformity

When uniformity was observed, 98.4% of CXM showed stain uniformity as compared with 96.8% of PIE slides. The difference was not statistically significant in terms of uniformity (P=0.133) suggesting that there was no difference in the two processing methods in producing uniform staining.

Pathologist A reported that 106(94.6%) of PIE and 111(99.1%) of CXM processed had consistency of stain. Pathologist B noted that 110(98.2%) of both CXM and PIE slides showed homogeneity. Evaluators C, D and E assessed presence of evenness on 110(98.2%), 107(95.5%) and 109(97.3%) of stained slides processed using PIE. They reported uniformity on stained slides in 111(99.1%), 109(97.3%), and 110(98.2%) of CXM. No statistical significant difference observed among the two methods (p>0.05) PIE and CXM slides were compared for invariableness of staining and the result showed agreement in 110,107, 111, and 108 by pathologists A, C, D and E respectively. Total of 18 mismatched pairs reported by the pathologists. Out of 12 mismatched pairs, 11 stained slides favored CXM slides while 07 favored PIE .NO mismatch reported by pathologist B. No statistical significant difference observed among the two methods (p>0.05) (Table 7)

Table 7: Table showing Uniformity of staining of conventional and PIE method

Pathologist	Conventional (n=112)			PIE(n=112)		**Significance**
		Number	**Percent**	**Number**	**Percent**	p-value
A	Present	111	99.1	109	97.3	(P=0.408)
	Absent	01	0.9	03	2.7	
B	Present	110	98.2	110	98.2	(P=1.000)
	Absent	02	1.8	02	1.8	
C	Present	111	99.1	107	95.5	(P=0.150)
	Absent	01	0.9	05	4.5	
D	Present	109	97.3	110	98.2	(P=0.701)
	Absent	03	2.7	02	1.8	
E	Present	110	98.2	106	94.6	(P=0.196)
	Absent	02	1.8	06	5.4	
Over all Total	Present	**551**	**98.4**	**542**	**96.8**	**(P=0.133)**
	Absent	**09**	**1.6**	**18**	**3.2**	



Fallopian tube (4x, 10x and 40x) - PIE method   Fallopian tube (4x, 10x and 40x) - CXM method

Figure 7: Photomicrography showing comparison of CXM & PIE processed tissues and H & E stainedsections for uniformity of staining

5.19.2. Clarity

When clarity was observed, 97.6% of CXM showed the devoid of cloudiness throughout the section with 95.3% of PIE (p=0.073).The difference was not statistically significant in terms of clarity of stained slides suggesting that there has no difference in the two processing methods in terms of producing non cloudy staining.

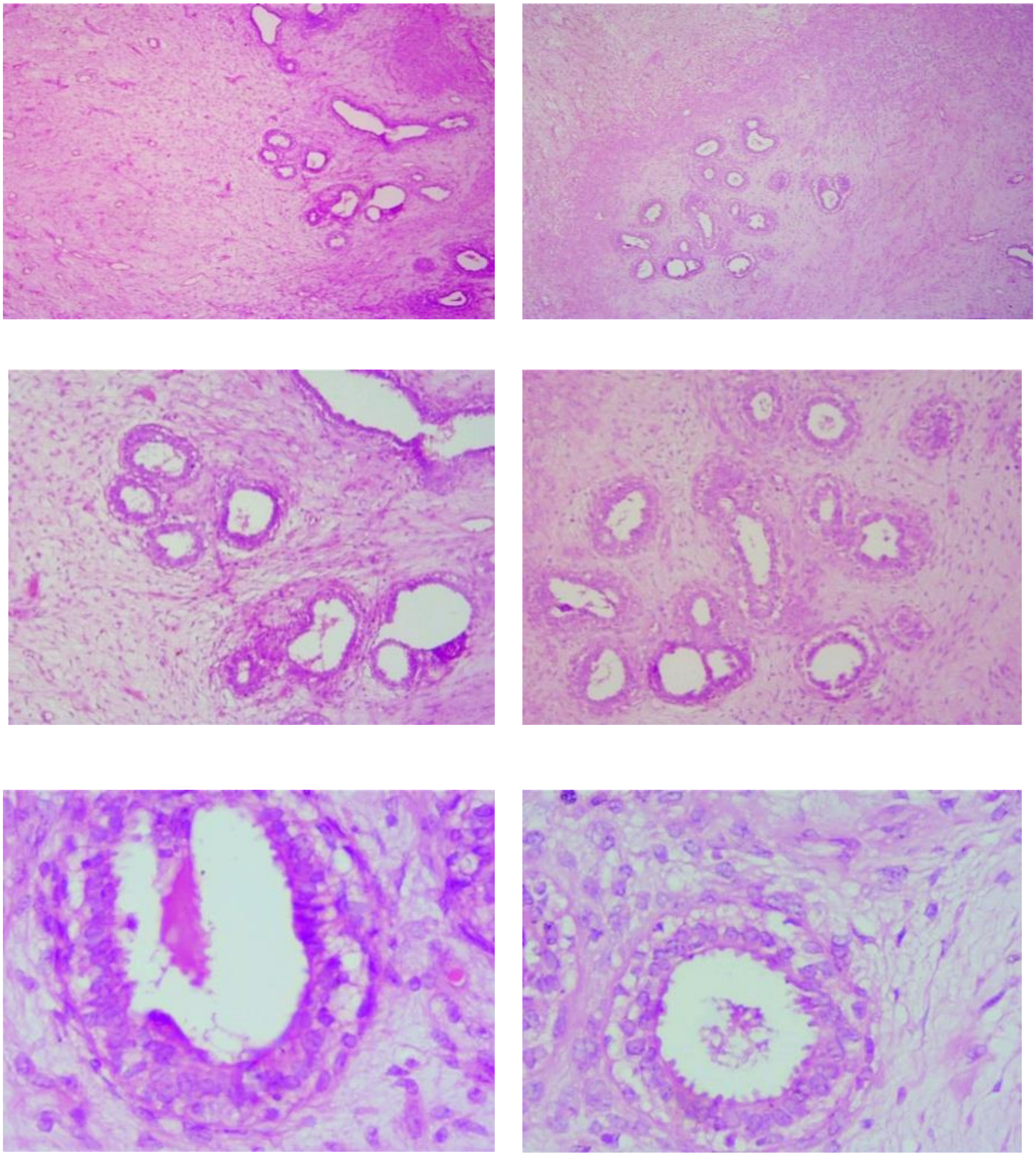
Section stained slides were reviewed by five pathologists to compare the presence or absence in the devoid of cloudiness throughout the section among method of tissue processing.

Pathologist A revealed 109 (97.3%) of CXM sections were free of cloudiness throughout the section while 105(93.7%) of PIE showed unambiguity .All slides of CXM were clear for pathologist B with only 108 (96.4%) of the PIE method. Pathologist C and E notified clarity in 109 (97.3%) and 110 (98.2%) of xylene processed tissues while 110 (98.2%) and 104(92.8%) of PIE assessed to be clear respectively. In terms of clarity no difference was reported by pathologist D (Table 8).

From 112 paired slides, pathologist A and B reported 08 and 04 untied pairs respectively. Only one discrepancy reported by pathologist C. No disagreement between the methods for clarity of staining indicated by Pathologist D. from the total 19 unpaired discrepancy 14 favored CXM while only one favored for PIE. (Table 8)

Table 8: Adequacy of Clarity of staining test in xylene and PIE processed tissues

Pathologist	Conventional (n=112)			PIE (n=112)		Significance
		frequency	**Percent**	**frequency**	**Percent**	**p-value**
A	Present	109	97.3	105	93.7	(P=0.235)
	Absent	03	2.7	07	6.3	
B	Present	112	100	108	96.4	(P=0.098)
	Absent	0	0	04	3.6	
C	Present	109	97.3	110	98.2	(P=0.701)
	Absent	03	2.7	02	1.8	
D	Present	107	95.5	107	95.5	(P=1.000)
	Absent	05	4.5	05	4.5	
E	Present	110	98.2	104	92.8	(P=0.075)
	Absent	02	1.8	08	1.2	
Over all Total	Present	**547**	**97.6**	**534**	**95.4**	**(P=0.073)**
	Absent	**13**	**3.4**	**26**	**4.6**	



Breast tissue -PIE method (4X, 10X, and 40 X) *Breast tissue processed using CXM method (4X, 10X, and40 X)*

Figure 8: Photomicrography showing comparison of CXM & PIE processed tissues and H & E stainedsections for clarity and crispness of staining

5.20.Crispness test

On analysis of crispness staining pattern, 97.5% of CXM showed well delineated nuclear membranes and sharply stained condensed chromatin against an unstained nucleolus as compared with 95.5% of PIE (p=0.108).There is no significant difference between the two processing methods in terms of crispness of stain, suggesting that there was no difference in the two processing methods in producing adequate crispness staining.

When crispness staining pattern noticed, pathologist A and B registered 104(92.8%) and 106(94.6%) of PIE processed and sectioned slides as'' adequate' 'respectively .These pathologists also mentioned that 109(97.3%) and 110 (98.2%) of CXM showed crispness. They also discovered method disagreements in nine unpaired slides (Five and four section slides by pathology A and B respectively).

Three pathologists (C, D and E) notified that 108(96.4%), 110(98.2%), and 109(97.3%) of the CXM slides exhibited crispness. They also stated that 106(96.4%), 108(97.3%), 109(96.4%) of the PIE had crispness respectively. It was reported to have only three unpaired slides disagreements among methods by pathologist (D and E).No disagreement between the methods for clarity of staining indicated by Pathologist C. From a total of fourteen unpaired slides discrepancies, eight of them were favored for CXM and four for PIE over CXM (Table 9).

Table 9: Adequacy of Crispness of staining test in xylene and PIE processed tissues

Pathologist	Conventional (n=112)			PIE(n=112)		Significance
		Number	Percent	Number	Percent	p-value
A	Present	109	97.3	104	92.8	P=0.153
	Absent	03	2.7	08	7.2	
B	Present	110	98.2	106	94.6	P=0.196
	Absent	02	1.8	06	5.4	
C	Present	108	96.4	108	96.4	P=1.000
	Absent	04	3.6	04	3.6	
D	Present	110	98.2	109	97.3	P=0.701
	Absent	02	1.8	03	2.7	
E	Present	109	97.3	108	96.4	P=0.734
	Absent	03	2.7	04	3.6	
Over all Total	Present	**546**	**97.5**	**535**	**95.5**	**P=0.108**
	Absent	**14**	**2.5**	**25**	**4.5**	

5.21.Adequacy for diagnosis

5.21.1. Microscopy result summary

The results showed that of the 112 sections studied, 95.7% of the Group CXM slides showed adequate nuclear staining as compared with 95% of PIE**.** The difference was not statistically significant (P=0.601) suggesting that there was no difference in the two processing methods in producing adequate nuclear staining **[**Table 9].

Adequate cytoplasmic staining was noted in 96.1 of sections in CXM as compared with 96.4% PIE Processing. The difference wasn't statistically significant (P=0.772) suggesting that there was no difference with in the two processing methods in producing adequate cytoplasmic staining **[**Table 6].

Uniform staining was present in 98.4% of CXM and in 96.8% of PIE Processing .The difference was not statistically significant (P=0.133) suggesting that there was no difference in the two processing methods **[**Table 7].

The clarity with CXM sections was 97.6%as compared with 95.3% of clarity in PIE Processing

[Table 8]. No statistically significant difference wasn't noted with in the two processing methods followed (P=0.073)

A crisp staining was seen in 97.5%, of sections in CXM as compared with 95.5% in PIE Processing. The difference wasn't statistically significant (P=0.108) suggesting that there was no difference with in the two processing methods in producing adequate nuclear staining **[**Table 9]. From the statistical data there was no statistical difference observed among pathologists in terms of the two methods while evaluating the parameters.

5.21.2. Total evaluation (Score, evaluators record, and Leica methods)

Method evaluation for introducing new tissue processing also uses final scoring system. The score method record adequacy if the total score is greater or equal to three, otherwise it is inadequate for diagnosis. CXM and PIE scored 97% and 95.8% adequacy for diagnosis. There is no statistical difference between the two processing methods in terms of adequacy for diagnosis ($p>0.05$: $p=0.668$) [Table 10]

Slides were also evaluated for their adequacy of diagnosis and recorded by pathologists. Of all slides evaluated, 97.5% and 96.1% of CXM and PIE were adequate respectively. The result revealed no statistical difference between the two methods in terms of adequacy for diagnosis ($p>0.05$: $p=0.229$) [Table 11]

Most tissue processor and processing method inventors mostly used this technique. The prominent tissue processor manufacturing company, Leica-Australia, uses an evaluation system which takes the percentage average of cutting, mounting, block stability, physical quality and chemical staining quality.it stated that percentages below 50% are poor, 70%-80% possible to use but not outstanding, and those above 80% are excellent.

Substitution of the conventional method is excellent if the final score of the new method is above 80% for all the parameters listed above .In our study, the new method accounted final score of 97.2% and all parameters were above 80% which is an excellent range. The adequacy registered in 97.2% of PIE as compared to 98.5% CXM showed no statistical differences among methods in terms of adequacy for diagnosis indicting the two methods are equivalent. [Table 12]

The overall result, in both comparison and assessment techniques used to evaluate the efficacy of the new tissue processing method, affirmed that PIE method is equivalent (at par) to the CXM. (No statistical difference and quality discrepancy registered).

Table 10: Adequacy for diagnosis in PIE and CXM processed sections (Score of 0- 2= Inadequate for diagnosis, while 3- 5= Adequate for diagnosis)

Parameter	Status	CXM	PIE	Significance p-value
Nuclear Staining (average Percentage of evaluators)	Adequate	95.7	95	0.601
	Inadequate	4.3	5	
Cytoplasmic Staining (average Percentage of evaluators)	Adequate	96.1	96.4	0.772
	Inadequate	3.9	3.6	
Clarity (average Percentage of evaluators)	Adequate	97.6	95.3	0.073
	Inadequate	3.4	4.6	
Uniformity of staining(average Percentage of evaluators)	Adequate	98.4	96.8	0.133
	Inadequate	1.6	3.2	
Crispness (average Percentage of evaluators)	Adequate	97.5	95.5	0.108
	Inadequate	2.5	4.5	
Adequate for diagnosis	**Adequate**	**97**	**95.8**	**0.668**
	There is no significant difference between the two processing methods in terms of adequacy for diagnosis			

Table 11: Adequacy for diagnosis (by pathologists) in CXM and PIE processed tissues

Pathologist	Conventional (n=112)			PIE(n=112)		Significance
		Number	Percent	Number	Percent	p-value
A	Adequate	111	99.1	109	97.3	P=0.313
	Inadequate	01	0.9	03	2.7	
B	Adequate	110	98.2	106	94.7	P=0.150
	Inadequate	02	1.8	06	5.3	
C	Adequate	107	95.6	110	98.2	P=0.249
	Inadequate	05	4.4	02	1.8	
D	Adequate	108	96.5	109	97.2	P=0.0.408
	Inadequate	04	3.5	03	2.8	
E	Adequate	110	98.2	104	92.9	P=0.062
	Inadequate	02	1.8	08	7.1	
Total	**Adequate**	**546**	**97.5**	**538**	**96.1**	**P =0.229**
	There is no significant difference between the two processing methods in terms of adequacy for diagnosis					

Table 12: Comparison of CXM and PIE using Leica standard processing evaluation system

	Conventional(n=112)		PIE(n=112)	
Score Summary	**Adequate (%)**	**Inadequate (%)**	**Adequate (%)**	**Inadequate (%)**
Cutting	**94.4**	**5.6**	**94.5**	**5.5**
Mounting	**98.7**	**1.3**	**98.2**	**1.8**
Block stability on storage	**99.1**	**0.9**	**99.1**	**0.9**
Physical quality of section	**98.3**	**1.7**	**98.3**	**1.7**
Quality of staining (chemical)	**97**	**3**	**95.8**	**4.2**
Final Score	**98.5**	**1.5**	**97.2**	**2.8**

5.21.3. Turnaround time

Turnaround time tells how fast we arrived to the patient. The TAT on tissue processing and staining using PIE mixture and Xylene was recorded while doing. The TAT comparison of the conventional and the new methods shows CXM took 16:30 hrs and the new processing method can be finished in 14hrs for total processing. This shows the PIE processing reduced the duration of processing by 15.2% (2:30hrs).

6. DISCUSSION

Ease of sectionability for blocks can be affected by the clearing agent employed during tissue processing [58]. In the sectioning test, easy sectioning with good serial sections was observed in 94.5% of the PIE mixture -processed tissues as compared with 94.4% in the xylene-processed tissues (P=0.967).This showed that there is no significant difference among the two methods in terms of cutting. This finding is similar with clearing done using rose oil (100%), bleached palm oil(100%) but much better than Ultra clear (81.7 %) [44,35, 46]. Sectioning difficulty was observed in Coconut oil processed tissues whereas it was easy in case of Xylene processed ones. Hardening and shrinkage are major causes of cutting difficulty. Both were minimized in case of PIE method which was similar to the study done on coconut oil [37].

The reason behind easy sectionability of PIE processed tissues might be due to the fact that the density of light liquid paraffin oil (0.88gm/ml) is closure to human fat (0.918) which helps fat removal during clearing to be with displacement instead of dissolution. Another reason could be the temperature adjustment at 65^0c; which might increase kinetic energy and decrease viscosity there by smooth exchange of reagents in and outside the tissue. The other reason might be the increased number of clearing stations which induces concentration gradient reduction hence gradual exchange of reagents [65, 66].

Some 0.9% of tissues that exhibited cutting difficulty were found to be hard skin, nipple, in nature which might have been incompletely cleared. This is supported with the findings stating that difficulties during sectioning arose on fibrous tissues due to their heterogeneous nature. This is all due to differential shrinkage of the various elements of hard tissues during fixation and tissue processing [57].Such tissues may require prolong exposure time in PIE mixture to achieve complete clearing. Reprocessing was done for this tissue by increasing the time stay in light liquid paraffin to 2hrs.

Adequate tissue mounting with cohesive and properly flatten sections without sweating was recorded in 98.7% of the CXM and 98.2% of the PIE during floatation (P=1.000).This depicted that PIE method is equivalent with CXM in terms of mounting. Absence of residual solvent during clearing and complete processing of tissue might be the reason for good mounting.

Physical qualities of sections were assessed by histologists microscopically for the presence or absence of section disruptions (holes and tears), uniformity of sections and proper section adhesion with the slides. Section disruption (holes and tears) and thickness uniformity were reveled in 2.1% and 97.3% of both methods (p=1.000) respectively. This indicates that PIE has equal ability with the CXM in providing uniform and cohesive sections. Adhesion of section with the slide was 100% in CXM while 99.7% (p=1.000) which is not statistically significant difference. The overall Physical quality of section excluding stain quality revealed that the two methods were equivalent in terms of producing uniform sections, section adhesions and absence of disruptions. Prolonged treatment in clearing solution will make the tissue brittle. Conversely, if specimen is not cleared properly, the paraffin will not impregnate properly and will lead to distortion of tissue during sectioning which will be demonstrated in sections as holes or tears. Hence the result for PIE might be due to complete and not prolonged clearing [72].

Block quality was evaluated by assessing block tissue shrinkage and opacity. The extent to which block surface contracts (shrinks) after sectioning is a good indicator as to the quality of clearing during processing. An improper cleared tissue shrinks back in to the block due to evaporation of residual clearing solution [73]. CXM showed around 1 % block shrinkage and opacity which PIE did not show (p=1.000).This revealed that PIE has better storability as compared to CXM but not statistically significant.

The finding of this study was similar to the study done on coconut oil with (100%) transparency or no opacity but much more better than kerosene (67%).Another study done by Sudeendra P et.al in 2014 on 60 soft tissue specimens showed significant shrinkage in xylene sample compared to coconut oil sample, which were again similar to this finding. [33, 42].Tissue transparency obtained in this study with PIE clearing method could be the matching of refractive index of light liquid paraffin oil with that of the tissue proteins which reduces the scattering of light and increases the optical clearance of the tissue. The other factor could be the raised temperature itself allowing easy diffusion of alcohol out of the tissue and penetration of the oil into the tissue with less difficult.

All the 112 PIE processed blocks did not reveal shrinkage after a week. Absence of shrinkage on this method may be due to the fact that the liquid paraffin is non-volatile and the use of two changes pure liquid light paraffin oil in removing the alcohols. Slow increase in concentration of clearing agent, from mixed proportion up to pure paraffin oil, ensures complete removal of dehydrants. In addition, ability of the molten paraffin wax to easily displace liquid paraffin oil may also contribute to absence of shrinkage. The slow increase in concentration of clearing agent, light liquid paraffin oil, privileged absence of shrinkage and hardening through gradual replacement of the dehydrants with clearants.

Clearing enhanced when tissue fats are dissolved. Fats dissolve fast and completely when the temperature is increased. In this study the temperature was adjusted to 65^0c to facilitate clearing. Moreover, increase in temperature boosts kinetic energy of molecules and rate of diffusion, with a corresponding decrease in viscosity [57, 59].In this study rate of diffusion enhanced by increasing temperature of paraffin oil at 65^0c.This ensured that the alcohols in the clearing solution diffused out of the tissue rapidly by allowing the replacement of liquid paraffin oil as at the same rate eliminating shrinkage.

Evaluations of histology processing methods also require assessment of microscopic staining characteristics of stained slides. Nuclear and cytoplasmic staining, uniformity, clarity and crispness parameters of stained slides were evaluated with both PIE and CXM methods.

Adequate nuclear staining demonstrated in 95% of PIE mixture processed sections as compared with 95.7% of conventional xylene method(p=0.601). Difference was not statistically significant, suggesting that there was no difference in the two methods with regard to nuclear staining adequacy. The finding was similar with reports of several studies done on sesame oil (90%), cedar wood oil (90%), bleached palm oil (91.7%), bleached palm oil (100%), Kerosene (100%), and coconut oil (100%) [32,39, 33, 35, 69, 37]**.** However, this result was incomparable with studies done using n-heptane, (84 %) and ultra-clear (67%) [34,46]**.**The result difference might be due to the fact that there was complete dehydration and clearing during tissue processing, sufficient staining time given for hematoxylin, and complete removal of paraffin wax. It might also be due to inherent nature of used clearants (density of n-heptane=0.68gm/ml; light liquid paraffin, 0.88gm/ml, and where as the PH of ultra-clear is acidic PH. =6.7) sample size and type.

In this study cytoplasmic staining was evaluated. PIE method sections showed 96.4% adequacy of staining pattern as compared with 92% of the conventional H and E sections(p=772). No statistically significant difference was seen between the two methods, suggesting that PIE was equivalent to conventional Xylene methods in terms of cytoplasm staining. This result is almost equivalent to a study done on cedar wood oil (93.3%) in Mahatma Gandhi Post Graduate Institute of Dental Sciences, India and bleached palm oil (91.6%) but less than a study done on coconut oil (100%). The result obtained was much better than the results of studies done on kerosene (60%), n-heptane (87 %) and Ultra-clear (60.9%) as a clearing agent [46,34,33,35,37, 39]. This difference in cytoplasmic staining character may be due to the eosin used as alcohol based eosin was utilized in this study while other studies used aqueous based eosin .On the other hand the differentiation might have its own good contribution to the quality of cytoplasm stain in case of PIE method.

Evaluating uniformity, 96.8 % of PIE and 98.4% of CXM sections slides showed uniform staining pattern suggesting that PIE staining method is equivalent/at par with conventional Xylene method. This result was similar to a study of clearing done on cedar wood oil (93.3%) whereas it is greater than studies done using sesame oil,83.3%),kerosene,(73.3%),n-heptane(77%) and ultra-clear(67%)[39,32,33,34,46].

On analysis for crispness of staining pattern, the cytoplasm of the sections were showed crispness of staining as observed in 95.5% of PIE sections as compared with 97.5% of CXM method processed and H & E stained slides (p=0.108) indicating the equivalency among methods. This finding is equivalent to 100% and 83% kerosene and n-heptane respectively [33, 34]. No statistically significant difference was seen. The result of this new method was of course greater than a study on liquid dish soap in Navodaya Dental College, India [43].

Clarity, devoid of cloudiness throughout the section, exhibited 95.3% adequacy in sections processed using PIE as compared to 97.5 % for sections processed using xylene. (P=0.073). This result was in consistent with bleached palm oil (91.6%). But incomparable with 83.3%, 80%, and 63.5% were adequate using sesame oil, n-heptane, and ultra-clear as clearants respectively [35, 32, 34, 46].

When the time duration for the entire processing procedure, i.e.: turnaround time, in both the processing methods was considered, the whole processing procedure was completed in 14hrs using PIE method when compared to the Conventional xylene that grabbed 16:30hrs. [49].

When the scores were totaled, 95.8% of PIE slides found to be adequate for diagnosis as compared with the 97 % of conventional xylene method. This is almost equivalent to ultra-clear

(100%) but much better than bleached palm oil (88.9%), cedar wood oil, and coconut oil [46, 37, 39, and 43].

Leica system uses the percentage average of cutting, mounting, block stability, physical quality and chemical staining quality. In this study, the new method scored 97.2% and all parameters were above 80% which is an excellent range. This showed that PIE is an excellent substitute for xylene.

The score method record adequacy if the total score is three, otherwise it is inadequate for diagnosis. PIE and CXM scored adequacy for diagnosis in 95.8% and 97% (p=0.668) of slides. There is no significant statistical difference between the two processing methods in terms of adequacy for diagnosis. Hence, PIE clearing method is equivalent with xylene method.

Slides adequacy for diagnosis were directly determined by pathologists .Of all slides evaluated, 97.5% and 96.1% of CXM and PIE were adequate respectively. The result revealed no statistical difference between the two methods in terms of adequacy for diagnosis (p=0.229) .The result indicated that PIE is equivalent with CXM. Therefore, PIE method is equivalent with Xylene in clearing during tissue processing. Overall the three methods of adequacy evaluations showed ability of PIE mixture as tissue clearants during tissue processing.

This finding is similar to other studies done on cedar wood oil (100%) ultra-clear (100%), coconut oil (100%), but better than bleached palm oil (88.9%) [39,46,37,69]. The method of adequacy analysis also contributed for the percentage difference. These studies used only scale method which has gap in exactly assuring adequacy because in scale method if both nuclear and cytoplasm were inadequate and the rest three were adequate it will be recorded as adequate for diagnosis. However diagnosis could not be possible. That is why ultra-clear showed adequacy in 100% but very much inadequacy in individual parameters.

Therefore, the PIE method was at par in two things; firstly, in producing adequate sections for diagnosis without compromising the quality of the stain and secondly, in saving considerable amount of time, cost, and being eco-friendly.

7. LIMITATION OF THE STUDY

7.14.Limitations of the study

- ✓ It would have been good if different staining techniques and immunohistochemistry were tried
- ✓ Small samples were not included ,so including such samples like punch biopsy will give sound
- ✓ Including Measurement systems like morphometric to asses shrinkage was not possible

8. CONCLUSION AND RECOMMENDATION

8.14. Conclusion

The quality of sections and stain obtained by using PIE methods were all adequate for diagnosis. Histologists found the blocks easy to cut and store and pathologists assured higher adequacy in staining and diagnosis. From these results of the present study infer that substitution of the conventional xylene with PIE as a clearing agent during tissue processing gives good processed tissues, cut sections and histological slides.

PIE mixture is nontoxic, non-hazardous, non-flammable, biodegradable, easy to handle, and readily available.

Using PIE mixture for clearing during tissue processing improves turnaround and may minimize the TAT of tissue processing without losing the quality of the histological details.

Hence, the new PIE method can be used as a substitution for the noxious xylene as a clearing agent in routine tissue processing with benefits of time, and environmental friendly with equivalent or at par quality with xylene. It is therefore necessary for histology and pathology laboratories to use PIE mixture into their routine tissue processing.

8.15. Recommendation

From the findings of this study the following recommendations are forwarded to improve substitution of hazardous xylene with bio –friendly ones:

- ✓ FMOH, RHBs and hospitals should introduce the use of PIE mixture as a clearing agent instead of xylene while expanding pathology service centers in addition to practicing in existing centers of the country.
- ✓ The standard Material safety data sheet /MSDS/ of hazardous chemicals, like xylene, should be properly prepared and implemented in histopathology laboratories with regular supervision.
- ✓ Hospitals shall give special attention for pathology laboratories building and premises. There should be a way or system of consulting pathology professionals (associations) while building pathology centers. They also should provide special care and compensation for the histopathology professionals.
- ✓ As the PPM of xylene is low, Pathology laboratory centers should introduce the mechanism and device of measuring exposure of professional's to hazardous chemicals like xylene.
- ✓ Histopathology laboratory professionals should be oriented and trained about on how to use PIE mixture as alternative to xylene during tissue processing.
- ✓ Pathology substitution methods comparisons better use the three analysis methods for evaluating adequacy of slides for diagnosis as using only the scale method has its own discrepancy like a slide with poor cytoplasm and poor nuclear stained could be considered as adequate for diagnosis but this looks impractical.
- ✓ Further research is recommended for academicians on the use of PIE mixture as a clearing agent in tissue processing using immunohistochemistry and other special stains. Researchers should also see options in substituting xylene during staining and mounting.

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Annexes

ANNEXE I: Preparation of working solutions

10% Neutral Buffered Formalin 40% formaldehyde =100 ml Distilled water= 900 ml Sodium dihydrogen phosphate monohydrate= 4 gm Disodium hydrogen phosphate anhydrous =6.5 gm Mayer's Hematoxylin (H) 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 AR 50 g or

The hematoxylin, potassium alum, and sodium iodate are dissolved in the distilled water by warming and stirring, or by allowing standing 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% Alcoholic Eosin (E) Eosin 1% stock

Dissolve 1gm of eosin Y water soluble in 20ml of distilled water and 80ml of95% alcohol.

Eosin working

Stock Eosin - 1 Part

Alcohol 80% - 3 Parts

Add 0.5ml of acetic acid just before use per 100 ml

Bluing solution

Sodium bicarbonate 2.5 gm Distilled water 1000 ml 40% Ethanol alcohol

400ml of Absolute ethanol 600ml of distilled water 80% Ethanol alcohol 800ml of Absolute ethanol 200ml of distilled water 96% Ethanol alcohol 960ml of Absolute ethanol 40ml of distilled water PIE MIXTURE-I

Light liquid paraffin oil=500ml

Isopropanol =300ml Absolute ethanol=200ml PIE MIXTURE-II

Light liquid paraffin oil=700ml Isopropanol =200ml

Absolute ethanol=100ml

Annex II: PIE method-Tissue processing procedures

Fixation

Formalin

Dehydration

40% ethanol alcohol

80% ethanol alcohol

96% ethanol alcohol Absolute ethanol alcoholAbsolute ethanol alcoholAbsolute ethanol alcohol

Clearing

Liquid paraffin, Isopropanol and Ethanol mixture Liquid paraffin, Isopropanol and Ethanol mixtureLiquid Paraffin oil

Liquid Paraffin oil

Infiltration

Paraffin wax

Paraffin wax

Annex III: CXM- Tissue processing procedures

Fixation

10% Neutral Buffered Formalin 2hrs

Dehydration

70% Ethanol.................................... 11/2 hrs

80% Ethanol..................................... 11/2 hrs

96% Ethanol...................................... 11/2 hrs

Absolute alcohol I ....11/2 hrs

Absolute alcohol II .... 11/2 hrs

Absolute alcohol III...........................11/2 hrs

Clearing

Xylene I .... 11/2 hrs

Xylene II .... 11/2 hrs

Infiltration

Paraffin wax I.................................... 1hrs

Paraffin wax II 1hrs

Paraffin wax III 1hrs

Paraffin wax III 1hrs

Annex IV: Mayer's Heamatoxylin and Eosin (H & E) Staining Protocol

Deparaffinization

Rehydration

Water 3 min

Absolute alcohol I 3 min Absolute alcohol II 3 min

95% Ethanol 2 min Rinse in distilled water 2 min

Stain in Mayer's Hematoxylin 15 minRinse in running tap water 4 min

1 % ammonium hydrate…1min

Rinse in running tap water 5 min70% ethanol

Counter stain in Eosin 1 min

Dehydration

95% Ethanol 3 min

Absolute alcohol II 2 minAbsolute alcohol I 2 min Absolute alcohol I 2 min

Clearing: Xylene II 2 min

Annex V: Pathologist Checklist

Addis Ababa University, School of medicine, Post graduate programPathology department:

Consent form and check list:

This check list is prepared for pathologists in order to use for the research purpose only. I, the principal investigator, Natinael Berhane (BSc, MA), am MSc candidate of Addis Ababa University, school of medicine, Department of pathology. I am trying to evaluate the clearing Efficacy of PIE mixture (mixture of paraffin oil, Isopropanol and ethanol).The study will help to identify gaps in the histopathology tissue processing and suggest for improvement.

True and direct information is needed while filling the check list. Your participation is highly valuable to address the issue. However, your participation is purely voluntary .All the information contained within the check list is tobe kept confidential.

I the undersigned with full understanding of the situation have given my consent voluntarily to the researcher to use the information gathered from me for the study. In addition, I have had the opportunity to ask questions about it and received clarification to my satisfaction.

Please direct any questions or problems you may encounter during this study to:Name: Natinael Berhane

Mobile: +251911957231,+251901918647

Email: natisisiye@gmail.com

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).

Pathologist's code--------

Block no	Nuclear staining	Cytoplasmic staining	Clarity of staining	Uniformity of staining	Crispness of staining	Adequacy for diagnosis	
						Adequate	Inadequate

Preference

Parameter	Equivalent	A	B
Slide preference			

Name_____________________________________sig__________________date____/
____/2020G.C

Annex VI: Histologist Checklist

Addis Ababa University, School of medicine, Post graduate program: Pathology department: Consent form and check list:

This check list is prepared for Histologists/histotechnologists in order to use for the research purpose only. I, the principal investigator, Natinael Berhane (MA, BSc), am MSc candidate of Addis Ababa University, school of medicine, Department of pathology. I am trying to evaluate the clearing Efficacy of PIE mixture (mixture of paraffin oil, Isopropanol and ethanol).The study will help to identify gaps in the histopathology tissue processing and suggest for improvement.

True and direct information is needed while filling the check list. Your participation is highly valuable to address the issue. However, your participation is purely voluntary .All theinformation contained within the check list is to be kept confidential.

I the undersigned with full understanding of the situation have given my consent voluntarily to the researcher to use the information gathered from me for the study. In addition, I have had the opportunity to ask questions about it and received clarification to my satisfaction.

Please direct any questions or problems you may encounter during this study to:Name: Natinael Berhane

Mobile: +251911957231, +251901918647

Email: natisisiye@gmail.com

Data Collection format- check list to be filled by clinical histologists/

Histologist code-----------

B.NO	**Block storage quality**		**Sectioning/cutting/ quality**				**Mounting**		
	shrinkage	**Opacity**	**Ribbon**	**compression**	**Cohesiveness**	**Uniformity**	**Dehydration and clearing/ Sweating/**	**flattening**	**cohesiveness**

Preference

Parameter	Equivalent	A	B
Block preference			

Name____Sig____________Date____/____/2020G.C

Annex VII. Declaration

I, the undersigned, declare that this MSc thesis is my original work, which has not been Presented for a degree in this or any other University and that all sources of materials used forthe thesis have been duly acknowledged.

MSc. Candidate: - Natinael Berhane (BSc, BA,MBA)

Signature:__________________________________Date of submission: ___________________

This thesis has been submitted with our approval by advisors;

Yonas Bekuretsion (MD, Pathologist, Associateprofessor,Addis Ababa University)

Signature:__________________________________Date________________Place: Addis Ababa, Ethiopia

Yeshwas Abite (PhD-Candidate,Addis Ababa University)

Signature:_______________ Date__________ Place: Addis Ababa, Ethiopia