Original Article

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Pathology Section

Assessment of the Effectiveness of Xylene-free Haematoxylin and Eosin Staining versus the Conventional Approach: A Comparative Observational Study

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ABSTRACT

Introduction: Xylene, a commonly used hydrocarbon in histopathological laboratories, poses significant risks, including carcinogenicity. Identifying safer alternatives is essential for laboratory safety and environmental sustainability.

Aim: To evaluate the feasibility and effectiveness of xylene-free Haematoxylin and Eosin (H&E) staining as a safer and more environmentally friendly alternative to conventional xylene-based staining methods.

Materials and Methods: A comparative observational study was conducted in the Department of Pathology at Karpaga Vinayaga Institute of Medical Sciences and Research Centre (KIMS & RC), Chengalpattu, Tamil Nadu, India, over a duration of three months (January 2024 to March 2024). The study included 50 specimen blocks (100 slides), with two sections taken from each block: group A (conventional staining) and group B (xylene-free staining). The 1.7% dishwashing liquid solution used in the xylene-free method was prepared by mixing 25 mL of commercially available dishwashing solution with 1,500 mL of distilled water. The staining quality, cellular architecture and overall staining effectiveness were analysed statistically using Statistical Package for Social Sciences (SPSS) version 25.0.

Results: The analysis shows that 49 (98%) of the slides in group A and 36 (72%) of the slides in group B had distinct nuclei and cytoplasm. The analysis of staining quality indicated that 45 (90%) of group A slides had good quality, whereas only 25 (50%) of group B slides achieved this standard. Overall quality was satisfactory in 49 (98%) of group A slides and only 36 (72%) of group B slides.

Conclusion: Xylene-free H&E staining is a viable alternative with comparable diagnostic quality and improved safety and environmental benefits. However, slight reductions in staining clarity suggest a need for further optimisation. Future research should focus on improving deparaffinisation efficiency and refining methodologies to enhance nuclear and cytoplasmic clarity while maintaining safety and sustainability benefits.

Keywords: Deparaffinisation, Histological techniques, Staining and labelling

INTRODUCTION

The fundamental steps in histopathology tissue processing for light microscopy are fixation, dehydration, clearing, infiltration with paraffin, followed by deparaffinisation and staining. Xylene, which has the same refractive index as proteins, is the most commonly used clearing agent. It acts by displacing alcohol, allowing maximum infiltration of paraffin. The clearing step renders the tissue sections translucent and clear, making them visible under a light microscope. Xylene also serves as a deparaffinising agent and has a paraffin solvent effect, facilitating the proper absorption of the H&E stain by the tissue sections [1].

Xylene is an aromatic hydrocarbon {dimethylbenzene, $C_6H_4(CH_3)_2$} obtained from petroleum and is naturally found in petroleum and coal tar. It is linked to adverse health effects, particularly among laboratory technicians and pathologists with prolonged exposure. Studies have documented that xylene inhalation can cause respiratory irritation, headaches, dizziness, neurological disturbances and dermatological reactions. Chronic exposure has been associated with potential carcinogenic risks, leading to strict safety regulations in laboratory environments. Furthermore, xylene is a Volatile Organic Compound (VOC) that contributes to air pollution and requires specialised waste disposal procedures to prevent environmental contamination [2].

To mitigate these risks, researchers have tested xylene-free alternatives, including essential oils (such as cedarwood oil), coconut oil, lemon water and commercial dishwashing liquid as substitutes. These alternatives aim to achieve comparable clearing and staining quality while eliminating the hazardous effects of xylene [3]. However, challenges such as variability in staining clarity, differences in tissue morphology preservation and occasional difficulties in deparaffinisation have been reported, necessitating further refinements in xylene-free protocols [4].

The shift toward xylene-free staining aligns with the broader objectives of improving laboratory safety, enhancing worker health and promoting sustainable practices in histopathology. By eliminating xylene exposure, laboratories can reduce occupational hazards, lower the incidence of respiratory ailments and minimise the risk of chemical-related illnesses among technicians and pathologists [5]. Additionally, xylene-free protocols simplify waste disposal, reducing the environmental burden associated with hazardous chemical handling. Research has shown that when optimised, xylene-free alternatives can provide diagnostic accuracy comparable to conventional methods while offering significant advantages in terms of safety and sustainability. As histopathology laboratories increasingly adopt ecofriendly and worker-safe solutions, xylene-free staining is emerging as a viable, cost-effective and health-conscious approach for routine diagnostic applications [6].

The present study aimed to evaluate the effectiveness of xylene-free H&E staining compared to the conventional xylene-based method and to study the effectiveness of xylene-free H&E staining of histopathology slides from specimens received in the Department of Pathology at KIMS & RC. By assessing staining quality, cellular architecture and overall diagnostic reliability, the study seeks to determine whether xylene-free staining can be a viable, safe and

sustainable alternative for routine histopathological diagnostics. The findings of the present study will contribute to enhancing laboratory safety, reducing environmental impact and optimising histopathological processing techniques for future applications.

MATERIALS AND METHODS

A comparative observational study was conducted in the Department of Pathology at Karpaga Vinayaga Institute of Medical Sciences and Research Centre (KIMS & RC), Chengalpattu, Tamil Nadu, India. The study was carried out over a period of three months, from January 2024 to March 2024. The study was conducted after obtaining approval from the Institution Ethics Committee, with IEC Ref No: KIMS/PG/09/03/2024 dated 09/03/2024. The study population comprised histopathological blocks and slides of specimens received in the Department of Pathology at KIMS & RC during the study period.

Sample size collection:

a. **Sampling frame:** Blocks stored in the Department of Pathology at study Institute.

b. **Sampling method:** Purposive sampling was chosen to ensure the inclusion of specimen blocks that allowed for the selection of cases with well-preserved histological features, facilitating a reliable assessment of staining quality and diagnostic accuracy. To minimise potential biases, multiple pathologists independently evaluated the slides and standardised staining protocols were followed.

Study tools: Blocks and corresponding histopathology slides.

Study Procedure

Data collection procedures: Data collection was conducted using the blocks available in the Department of Pathology. Two sections were taken from 50 representative blocks and sorted into two groups: group A (50 sections subjected to conventional staining) and group B (50 sections subjected to the xylene-free approach). Staining was performed according to their respective procedures.

Staining procedures:

Conventional staining approach: Paraffin sections were dewaxed using xylene, rehydrated through descending grades of alcohol to water and stained in alum haematoxylin for an appropriate duration. Sections were then washed in running tap water until they turned blue, differentiated in 1% acid alcohol for 5-10 seconds and washed again until re-blued. Following this, sections were stained with 1% eosin Y for 10 minutes, washed, dehydrated through ascending grades of alcohol, cleared using a xylene dip and finally mounted.

Xylene-free staining approach: This method utilises a 1.7% dishwashing liquid solution prepared by mixing 25 mL of commercially available dishwashing liquid with 1,500 mL of distilled water. The dishwashing liquid should ideally contain a high surfactant content with minimal additives, such as perfumes, dyes, or antibacterial agents, ensuring effective paraffin removal without interfering with staining quality. Sections were dewaxed in this heated solution at 90°C for five minutes, followed by a distilled water wash and rehydration through descending grades of alcohol. The subsequent staining steps mirrored those of the conventional method, except that clearing was performed using the same 1.7% dishwashing liquid instead of xylene before mounting.

The 1.7% concentration was selected to provide an optimal balance between efficient paraffin removal and tissue preservation. Higher concentrations could potentially cause excessive tissue damage or staining inconsistencies, while lower concentrations might be insufficient for complete dewaxing. This formulation has been explored as an effective and safer alternative to xylene in histological staining, with similar approaches reported in studies on surfactant-based dewaxing methods [7,8].

Handling and evaluation of slides: After staining, all slides were carefully handled to prevent contamination and ensure uniform assessment. The slides were mounted using Distrene Plasticizer Xylene (DPX) mountant, a commonly used synthetic resin that provides long-term preservation and enhances optical clarity. Once mounted, the slides were stored in labelled slide boxes to protect them from dust and physical damage before microscopic evaluation.

To ensure consistency and minimise variability in assessment, standardised protocols were followed during microscopic examination. All slides were evaluated under identical light microscope settings, including magnification (10×, 40× and 100× objectives) and illumination conditions. This standardisation helped reduce observer bias and ensured that staining quality and cellular architecture were assessed under uniform conditions. Additionally, evaluations were conducted by multiple pathologists to enhance the reliability and reproducibility of the findings.

Assessment of staining:

- Nuclei: Blue/black
- Cytoplasm: Varying shades of pink
- Muscle fibres: Deep pink/red
- Red blood cells: Orange/red
- Fibrin: Deep pink

Criteria for analysing the results of the staining procedure [1]: The quality of cellular architecture and staining quality was assessed in the slides under a light microscope.

1. Cellular architecture:
   - Score 0: Indistinct nucleus-cytoplasm
   - Score 1: Distinct nucleus-cytoplasm
2. Quality of staining:
   - Score 0: Poor
   - Score 1: Satisfactory
   - Score 2: Good
3. Overall quality of staining: Diagnostic accuracy was assessed based on the ability to distinguish nuclear and cytoplasmic details clearly. The overall quality score was calculated by summing the scores for cellular architecture and staining quality.
   - Total score of 0-1: Unsatisfactory
   - Total score of 2-3: Satisfactory

Gold standard: The gold standard for assessing diagnostic accuracy was the conventional xylene-based H&E staining method (group A). The slides stained using xylene-free methods (group B) were evaluated against this standard by multiple independent pathologists to ensure reliability and minimise observer bias.

STATISTICAL ANALYSIS

Data entry was performed using Microsoft Excel, followed by data cleaning and validation for consistency. Statistical analysis was conducted using SPSS software version 25.0. Continuous variables were presented as mean and standard deviation, while categorical variables were expressed as frequency and percentage. Results were displayed through tables and figures. The Chi-square test was used for comparing categorical variables, with a p-value >0.05 considered statistically significant.

RESULTS

In the present study, slides were examined for cellular architecture based on the presence of distinct nuclei and cytoplasm in the tissue. About 49 (98%) of the group A (Conventional staining) slides exhibited distinct nuclei, whereas only 36 (72%) of the group B slides (Xylene-free staining) displayed distinct features, as tabulated in [Table/Fig-1].

Cellular architecture	Conventional staining Group A (n=50) n (%)	Xylene-free approach Group B (n=50) n (%)
Indistinct nucleus- cytoplasm	1 (2)	14 (28)
Distinct nucleus- cytoplasm	49 (98)	36 (72)

[Table/Fig-1]: Distribution of cellular architecture between slides subjected to conventional staining approach (group A) and Xylene-free staining approach (group B) N=100.

Analysis of staining quality indicates that 22 (44%) of the slides in group B were assessed as having satisfactory staining compared to only 4 (8%) in group A. Additionally, good staining was observed in 45 (90%) of group A slides, but this was lower in group B, with only 25 (50%) achieving a good rating, as shown in [Table/Fig-2]. Group B demonstrated a slightly higher percentage of satisfactory staining despite having more indistinct cellular architecture, which may be attributed to a more even distribution of eosin, enhancing cytoplasmic contrast and overall appearance.

Quality of staining	Conventional staining Group A (n=50) n (%)	Xylene-free approach Group B (n=50) n (%)
Poor	1 (2)	3 (6)
Satisfactory	4 (8)	22 (44)
Good	45 (90)	25 (50)

[Table/Fig-2]: Distribution of quality of staining between slides subjected to conventional staining approach (group A) and Xylene-free staining approach (group B).

The overall quality of each slide was categorised as satisfactory or unsatisfactory, as detailed in [Table/Fig-3]. Satisfactory staining quality was observed in 49 (98%) of the group A slides, while only 36 (72%) of the group B slides received a satisfactory rating. This is evidenced by the presence of good cellular architecture with distinct nuclei and cytoplasm, as illustrated in [Table/Fig-4a,b]. In contrast, unsatisfactory quality was higher in group B, with 14 (28%) compared to 1 (2%) in group A. This lack of cellular architecture in group B was reflected by indistinct nuclei and cytoplasm, as shown in [Table/Fig-4c,d].

Overall quality	Conventional staining Group A (n=50) n (%)	Xylene-free approach Group B (n=50) n (%)
Satisfactory	49 (98)	36 (72)
Unsatisfactory	1 (2)	14 (28)

[Table/Fig-3]: Distribution of overall quality between slides subjected to conventional staining approach (group A) and Xylene-free staining approach (group B).

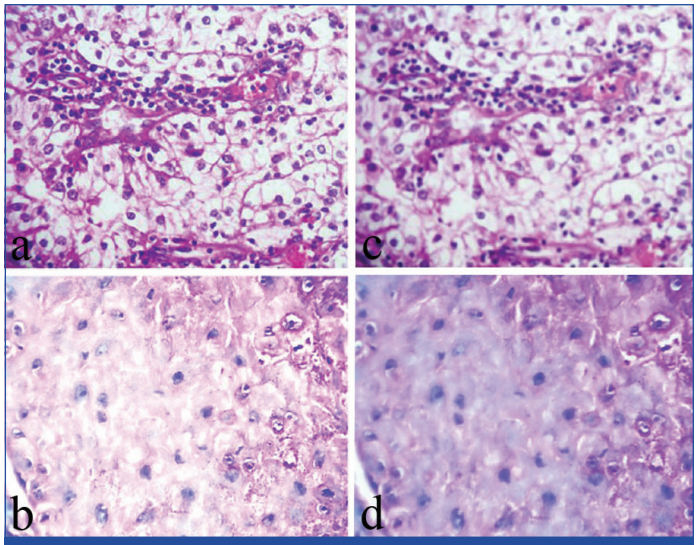
A comparison of the conventional staining approach and the xylene-free staining approach between the two groups is presented in [Table/Fig-5]. The quality of staining (t=2.71, p-value=0.0103*) and overall quality (t=2.58, p-value=0.0142*) showed statistical significance.

DISCUSSION

The present comparative observational study included 50 specimen blocks (100 slides), with two groups derived from each block: group A (conventional staining) and group B (xylene-free staining). The findings of the present study indicate that although the conventional staining method provided superior cellular architecture and overall staining quality, the xylene-free method offered comparable staining quality with improved safety and reduced environmental impact. This suggests that the xylene-free approach is a viable alternative to the conventional method, providing relatively similar diagnostic accuracy with enhanced safety benefits.

Cellular Architecture

The present study shows that distinct nuclei and cytoplasm were significantly higher in group A [Table/Fig-1], with 49 (98%) compared to 36 (72%) in group B. This is consistent with the study conducted



[Table/Fig-4]: a) Clear cell renal carcinoma, H&E (40x) (conventional staining method). Good cellular architecture (distinct nucleus and cytoplasm) and satisfactory quality of staining noted {Total score=3 (1+2)}; b) Squamous cell carcinoma, H&E (40x) (conventional staining method). Satisfactory quality of staining was evident with distinct nucleus and cytoplasm [Total score=2 (1+1)]; c) Clear cell renal carcinoma, H&E (40x) (Xylene-free staining method). Cellular architecture was unsatisfactory though the quality of staining was satisfactory {Total score=1 (0+1)}; d) Squamous cell carcinoma, H&E (40x) (Xylene-free staining method). Unsatisfactory quality of staining with indistinct nucleus and cytoplasm[Total score=0 (0+0)].

S. No.	Variables	Group A (n=50) (Mean±SD, 95% CI)	Group B (n=50) (Mean±SD, 95% CI)	t-value	p-value
1.	Cellular architecture	1±0.141 (0.96-1.04)	1±0.454 (0.87-1.13)	1	0.324
2.	Quality of staining	2±0.385 ((1.89-2.11)	1±0.611 (0.83-1.17)	2.71	0.0103*
3.	Overall quality	3±2 ((2.45-3.55)	0.495±0.976 (0.22-0.77)	2.58	0.0142*

[Table/Fig-5]: Comparision of conventional staining approach and Xylene-free staining approach between two groups.
*p-value <0.05 is statistically significant

by Suvarna KS et al., in which xylene was identified as an effective clearing agent, facilitating optimal nuclear detail and cytoplasmic contrast [9]. About 14 (28%) of the slides in group B [Table/Fig-1] exhibited indistinct nuclear-cytoplasmic borders, in contrast to 1 (2%) in group A. This suggests that, while xylene-free protocols may effectively preserve overall tissue morphology, they may not adequately provide the necessary nuclear and cytoplasmic clarity.

Staining Quality and Diagnostic Accuracy

The present study indicates a significant difference in staining quality between the two groups. In group B, 22 slides (44%) exhibited satisfactory staining, compared to 4 slides (8%) in group A. However, the proportion of slides with good staining was notably lower in group B [Table/Fig-2], with 25 slides (50%) as opposed to 45 slides (90%) in group A [Table/Fig-2]. Similar results were reported in a study conducted by Sravya T et al., who found satisfactory staining quality in 44% of xylene-free slides. Their findings support the potential of xylene-free methods as a viable alternative, provided modifications are implemented to enhance the clarity of cellular architecture [10]. The slightly higher percentage of satisfactory staining observed in group B in the present study may be attributed to the uniform distribution of eosin, which enhances cytoplasmic contrast and overall stain appearance.

Potential Optimisations for Xylene-free Staining

Modifications in xylene-free protocols, such as using alternative clearing agents like cedarwood oil or coconut oil, could further enhance staining quality, as suggested in a study conducted by Indu S et al. This study supports xylene-free staining as a viable alternative, balancing diagnostic efficiency with enhanced safety

benefits [11]. The overall quality of staining in the present study was 49 (98%) in group A compared to 36 (72%) in the xylene-free group. This indicates that, although pathologists were able to make reliable diagnoses using the xylene-free method, there are challenges in implementing xylene-free staining in histopathology labs, including workflow adjustments, equipment considerations and training requirements to improve cellular architecture and staining clarity. Similarly, a study by Ramaswamy AS and Dayasagar P suggested the modification and optimisation of the staining methodology [12]. This suggests the use of alternate clearing agents, adjustments in the concentration of dishwashing liquid to improve deparaffinisation efficiency and stain penetration, extended rehydration time in alcohol or water and the use of buffering solutions to eliminate non uniformity of staining, improve staining clarity and preserve cellular integrity.

Although statistical significance was observed in staining quality and overall quality (p-value=0.0103* and p-value=0.0142*, respectively), it is essential to assess the clinical impact of these differences. The slightly lower nuclear clarity in xylene-free staining could affect subtle diagnostic interpretations, particularly in cases requiring precise nuclear detail, such as dysplasia or malignancy. However, for routine histopathological diagnoses, the observed differences may not significantly alter diagnostic outcomes.

Safety and Environmental Impact

Xylene is a well-known hazardous substance, with exposure linked to various health issues, including respiratory problems and skin irritation. The adoption of xylene-free alternatives not only minimises these risks but also contributes to a safer working environment. The environmental impact of xylene has also been a concern, leading researchers to explore ecofriendly alternatives. The study by Rajan ST and Malathi N underscored the environmental effects of xylene, the toxic effects of xylene exposure on the body and the importance of proper disposal of xylene [13].

Operational Efficiency

Operational efficiency is another critical factor positively influenced by the shift to xylene-free staining methods. A study by Pandey P et al., noted that xylene-free protocols tend to be relatively faster, reducing overall processing time by approximately 30-40% [14]. This increase in efficiency can be particularly beneficial in high-throughput histopathology laboratories, where time savings can lead to increased productivity without sacrificing staining quality.

Economic Considerations

While the initial cost of switching to xylene-free protocols might be a concern, long-term economic benefits can be realised through reduced costs associated with health and safety compliance, chemical disposal and potentially lower insurance premiums due to decreased occupational hazards. A study by Swamy SR et al., highlighted the effectiveness of inexpensive, less toxic and easily available xylene substitutes for H&E staining [15].

Limitation(s)

Variability in study design, methodologies and staining protocols challenges the standardisation and generalisability of xylene-free methods. The smaller sample size (50 blocks, 100 slides) and the sampling method (purposive sampling) limit the findings of the study. This limitation can be addressed by expanding the study across multiple institutions to validate the findings, assess interobserver variability and refine the protocols for different tissue types. Although the initial cost of xylene-free methods may be higher, the long-term benefits may outweigh these costs.

CONCLUSION(S)

The shift towards ecofriendly alternatives, such as dishwashing liquid and essential oils, aligns with global sustainability goals and promotes responsible laboratory practices. In the coming years, xylene-free staining is poised to become the standard approach, offering a balanced solution that enhances laboratory safety, reduces environmental impact and maintains high diagnostic accuracy. Conducting an economic analysis of the long-term benefits would help assess the feasibility of adopting xylene-free methods in various settings, ensuring cost-effectiveness and sustainability. Further research is needed to standardise protocols and evaluate long-term cost implications. Studies focusing on large-scale implementations in various settings could provide additional insights.

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