Research Article

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Xylene and Alcohol-Free Method for Histological Preparation: An Experimental Study

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ABSTRACT

Background: Liquid dish washing soap (DWS) presents as a new substitute for deparaffinizing in hematoxylin and eosin (H and E) staining method. Objective of the study was to evaluate and compare the quality of xylene alcohol free (XAF) deparaffinized sections with liquid DWS to that of conventional H and E sections.

Materials and Methods: Study included sections from thirty paraffin embedded tissue blocks, each stained by conventional H and E and XAF H and E staining method. Scoring was done on the basis of five parameters-nuclear staining, cytoplasmic staining (adequate = score1, inadequate = score0), clarity, uniformity and crispness of staining (present = score1, absent = score0). Score >/= 2 was inadequate for diagnosis and 3-5 was adequate for diagnosis. Statistical analysis was done using Z test.

Results: Liquid DWS sections scored better for crispness (76.6%) and clarity (86.6%). Nuclear staining (96.6%), cytoplasmic staining (100%) and uniformity of staining (100%) were similar in both sections. 96.6% H and E sections stained adequately for diagnosis when compared with 100% in liquid DWS sections.

Conclusion: Liquid DWS is a safer, non toxic, alternative and effective substitute to xylene in routine H and E staining procedure.

Keywords: Deparaffinization, liquid dish washing soap, xylene alcohol free.

INTRODUCTION

Basic objective in any field of life sciences is the utilization of innocous, economical, less bio-hazardous and eco-friendly chemicals and materials, particularly in medical and histology laboratory.[1] H and E staining procedure, considered as the gold standard, is the most popular staining method. Even after 150 years of its introduction, it is perpetual.[2] Xylene and alcohol used in the conventional procedure have various ill-effects, making it a potential occupational hazard. Demerits of xylene and alcohol comprises of toxicity, polluted working environment, hazardous disposal and cost containment.[3]

Different cognate reagents: limonene reagents, vegetable oil, olive oil, aliphatic and aromatic hydrocarbons and mineral oil, have been used as substitute of xylene for deparaffinizing. [1] Falkeholm *et al.* in 2001, for the first time attempted the use of liquid dish washing soap (DWS) for deparaffinizing the tissue sections by eliminating both xylene and alcohol from H and E staining procedure.[3] The present study is intended to utilize liquid dishwashing solution as a substitute for xylene and alcohol in dewaxing and staining without impairing the morphology, diagnostic

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values as well as the staining characteristics of the tissue sections.[2]

AIMS AND OBJECTIVES

- To analyze the hypothesis that xylene and alcohol free (XAF) H and E sections deparaffinized with diluted DWS are better than or equivalent with the conventional H and E sections.
- To assess and inspect the quality of XAF H and E sections with that of the conventional H and E sections.

MATERIALS AND METHODS

Tissue samples:

The present prospective study was carried out on 30 paraffin-embedded tissue blocks of various specimens submitted in the Department of Oral and Maxillofacial Pathology.

Reagents used:

Commercially available liquid dish washing soap (DWS) was used as an alternative for xylene and alcohol. 25 ml of the liquid DWS diluted in 1500 ml of distilled water was used.

Histological procedure:

Two sections of 4µm each were cut from the tissue blocks. One section was stained with conventional H and E staining method (Group A) and the other with XAF H and E staining method (Group B).

Conventional H and E staining method (Group A):

1. Xylene 1- 5 minutes
2. Xylene 2- 5 minutes
3. 90% alcohol – 5 minutes
4. 70% alcohol – 5 minutes
5. Water wash – 5 minutes
6. Harri's hematoxylin – 5 minutes
7. Water wash – 5 minutes
8. 1% acid alcohol – 1 dip
9. Water wash – 5 minutes
10. 1% eosin – 1 dip
11. Water wash- 1 minute
12. 70% alcohol – 5 minute
13. 90% alcohol – 5 minute
14. Xylene 1- 5 minutes
15. Xylene 2- 5 minutes
16. Oven dry the section at 60°C for 2 minutes
17. Mount with DPX and cover slip

XAF H and E staining method (Group B):

1. Diluted DWS 1- at 90 ^{0}C 1 minute
2. Diluted DWS 2- at 90 ^{0}C 1 minute
3. Distilled water 1 – at 90 ^{0}C 30 seconds
4. Distilled water 2 – at 90 ^{0}C 30 seconds
5. Distilled Water 3 – at 45 ^{0}C 30 seconds
6. Distilled water 4- at room temp. for 30 seconds
7. Harri's haematoxylin – 5 minutes
8. Water wash – 5 minutes
9. 1% acid alcohol – 1 dip
10. Water wash – 1 minute
11. 1% eosin – 1 minute
12. Water wash – 1 minute
13. Oven dry the sections at 60 ^{0}C for 2 minutes
14. Mount with DPX and cover slip

Assessment of stained sections:

30 tissue sections stained with conventional H and E staining method (group A) and 30 sections stained with the XAF H and E staining method (group B) were coded.

Based on the trailing parameters, slides were assayed using the scoring system of *Ankle et al* (2011).[1]

1. Nuclear staining (adequate = score 1, inadequate = score 0)
2. Cytoplasmic staining (adequate = score 1, inadequate = score 0)
3. Clarity of staining (present = score 1, absent =score 0)
4. Uniformity of staining (present = score 1, absent = score 0)
5. Crispness of staining (present = score 1, absent = score 0)

Scoring of the sections:

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

Table 1: Comparative scores for each parameter obtained in Group A and Group B sections.

Parameter	Conventional H&E staining (Group A)	XAF H&E staining (Group B)	Z value	P value	Significance
Nuclear staining Adequate Inadequate	29 (96.6%) 01 (3.3%)	29 (96.6%) 01 (3.3%)	0	1	Not significant
Cytoplasmic staining Adequate Inadequate	30 (100%) 0 (0 %)	30 (100%) 0 (0 %)	0	1	Not significant
Clarity Present Absent	22 (73.3%) 08 (26.6%)	26 (86.6%) 04 (13.3%)	1.2472	0.2113	Not significant
Uniformity Present Absent	30 (100%) 0 (0%)	30 (100%) 0 (0%)	0	1	Not significant
Crispness Present Absent	17 (56.6%) 13 (4.3%)	23 (76.6%) 7 (23.3%)	1.6352	0.001	Significant

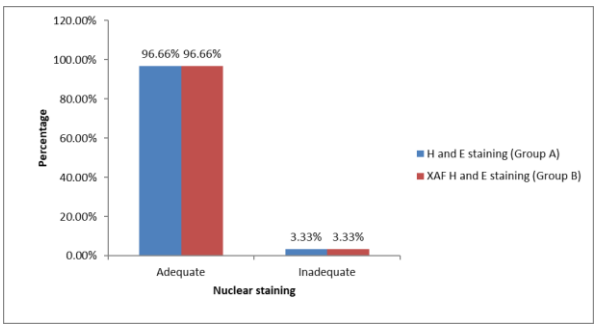
'P' value less than 0.05 was considered as significant and vice versa.

Table 2: Score for adequacy for diagnosis for both group sections.

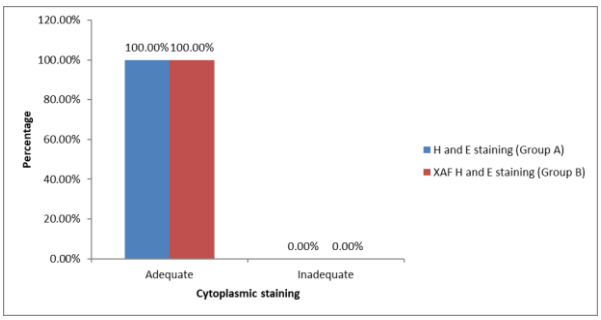
Score	Normal H&E staining (Group A)	XAF H&E staining (Group B)	Z value	P value	Significance
Adequate for diagnosis	29 (96.6%)	30 (100%)	1.1066	0.267	Not significant
Inadequate for diagnosis	01 (3.3%)	0 (0%)	1.1066	0.267	Not significant

'P' value less than 0.05 was considered as significant and vice versa.

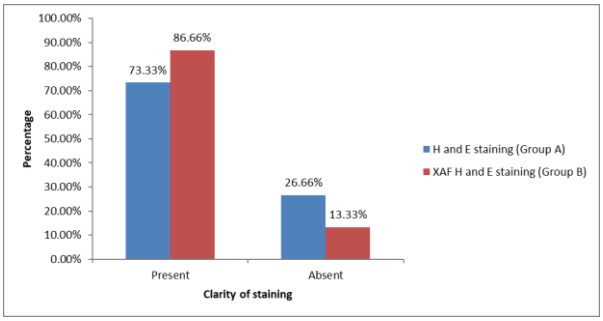
After application of scores, following findings were depicted. Adequate nuclear staining 96.66% [Graph 1], adequate cytoplasmic staining 100% [Graph2] and uniform staining 100% was present in both groups (Z=0, $P > 0.05$) [Graph 4]. Clarity was present in 73.33% of group A and in 86.66% of group B sections (Z= 1.247, $P > 0.05$) [Graph 3, Figure 1,2,3] and crisp stain was noted in 56.66% of group A and 76.66% of group B sections (Z= 1.635, $P < 0.05$) [Graph 5, Figure 3]. The differences observed between the parameters in both the groups except crispness were not statistically significant [Table 1]. The staining was found to be adequate for diagnosis in 96.66% of group A sections as compared with 100% of group B sections (Z=1.106, $P > 0.05$) [Table 2] but the difference was not statistically significant.



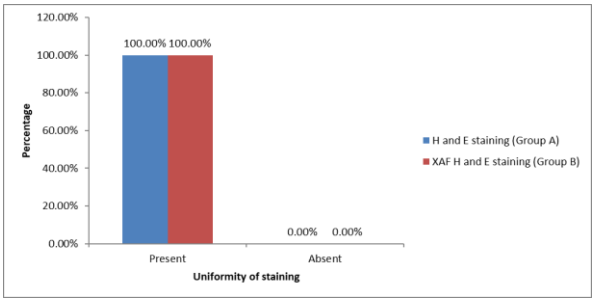
Graph 1: Comparison of adequacy of nuclear staining in group A and B.



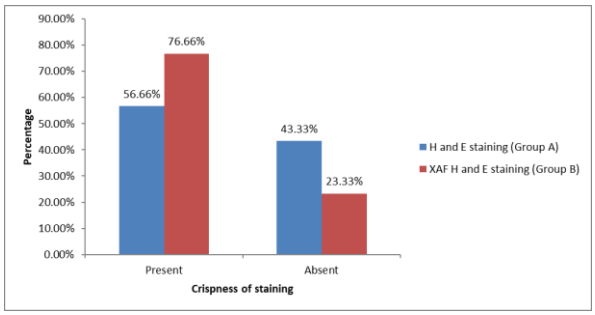
Graph 2: Comparison of adequacy of cytoplasmic staining in group A and B.



Graph 3: Comparison of clarity of staining in group A and B.



Graph 4: Comparison of uniformity of staining in group A and B.



Graph 5: Comparison of crispness of staining in group A and B.

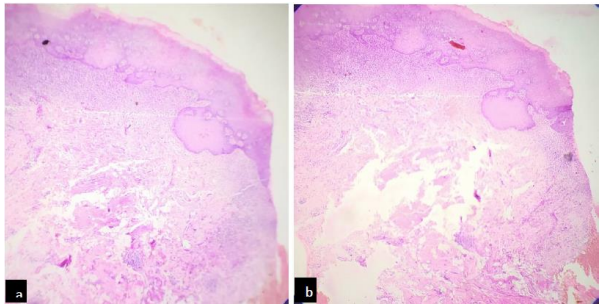


Fig 1: Photomicrograph showing clarity of staining in tissue. (a) conventional H and E stain (4x) (b) Xylene alcohol free (XAF) stain (4x).

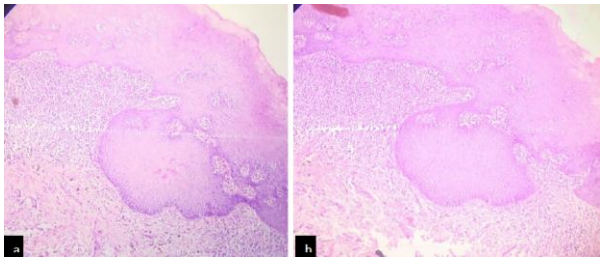


Fig 2: Photomicrograph showing clarity of staining in tissue. (a) conventional H and E stain (10x) (b) Xylene alcohol free (XAF) stain (10x).

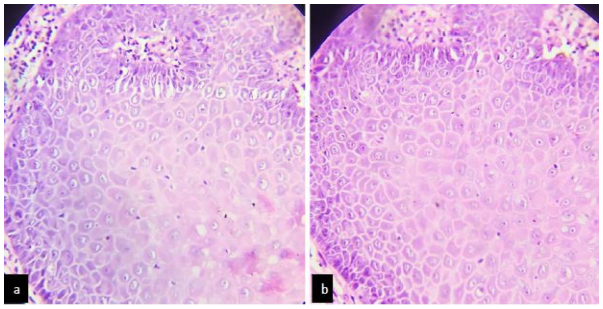


Fig 3: Photomicrograph showing clarity and crispness of staining in tissue. (a) conventional H and E stain (40x) (b) Xylene alcohol free (XAF) stain (40x).

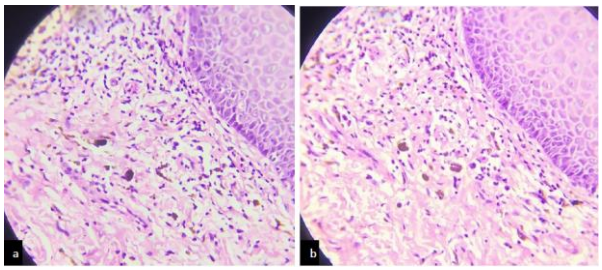


Fig 4: Photomicrograph showing difference of staining in melanin pigment. (a) conventional H and E stain (40x) (b) Xylene alcohol free (XAF) stain (40x).

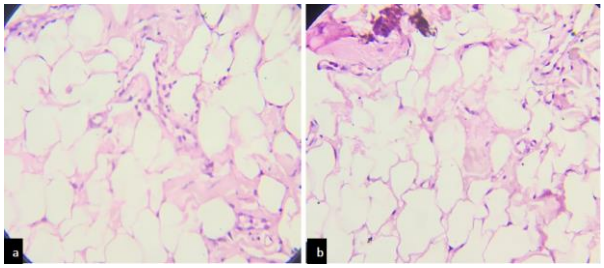


Fig 5: Photomicrograph showing difference of staining adipose tissue. (a) conventional H and E stain (40x) (b) Xylene alcohol free (XAF) stain (40x).

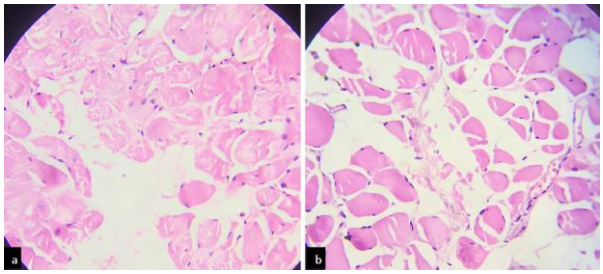


Fig 6: Photomicrograph showing difference of staining in muscle fibre. (a) conventional H and E stain (40x) (b) Xylene alcohol free (XAF) stain (40x).

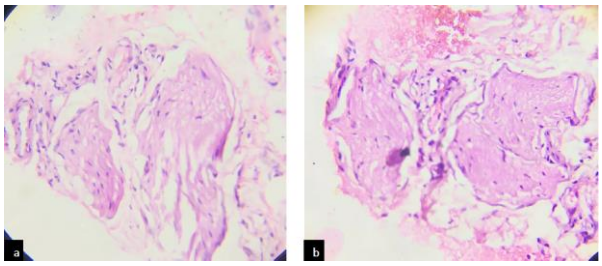


Fig 7: Photomicrograph showing difference of staining in nerve fibre. (a) conventional H and E stain (40x) (b) Xylene alcohol free (XAF) stain (40x).

Table 3: Advantages of XAF H and E staining over conventional H and E method.

	Conventional H and E	XAF H and E
Expenditure	High	Low
Duration	70-80 min	30-35min
Toxicity	Present	Absent
Biohazardous	Yes	No
Combustion of chemicals used	Present	Absent
Staining protocol	Lengthy	Simplified
Handling	Toxic if not properly handled	Easy
Nature of staining	Good	Good
Disposition of chemicals	Difficult	Easy

DISCUSSION

Histopathological diagnosis of various lesions by the examining the biopsy specimens submitted, requires the preparation of sections from paraffin embedded tissue blocks. It includes various steps like fixation, dehydration, clearing, impregnation and embedding.[10] After the sections are snipped with microtomes, they are dewaxed and hydrated before they are finally stained with Hematoxylin and eosin stains. Xylene and alcohol forms an inseparable part of a histopathology laboratory. Histopathological technicians by virtue of the nature of their work are maximally exposed to xylene. Existing legitimate exposure limit for xylene is 100ppm as an 8 hour time weighted average (TWA) concentration as per Occupational Safety and Health Administration [4] Developed nations have stringent guidelines for their usage, however in India, the usage of such chemicals is slack and lacks standardization. This leads to an indirect impact on the safety of the workers handling these chemicals. In this context, any technique which could minimize or eliminate the use of xylene as well as alcohol without compromising on the stain quality or time for processing would be much appreciated.

Various research works published by Buesa et al. (2009), Ankle and Joshi et al (2011), Ramulu et al (2012), Pandey et al (2014) have also used liquid dish washing detergent as a substitute for xylene in deparaffinizing sections in XAF H & E staining and concluded XAF technique was at par with conventional H & E staining procedure.[5] In contrast, Ramaswamy at el (2017) observed that XAF method failed to prove that it has comparable effects as that of conventional method.[10]

This experimental study focuses on observing and evaluating a new deparaffinizing agent in H and E staining method, i.e. the liquid dish washing soap (DWS), as an auxiliary to xylene and alcohol and there by rejuvenating the technique which is less toxic, time-saving and cost-effective. The advantages of XAF H and E staining over the conventional one are elucidated [Table 3].[1]

The liquid dish washing detergent is taken as a substitute as it performs the same function (deparaffinization) as that of xylene and when used in meager amounts (2%), it has less chances of being toxic when compared to the harmful effects of xylene.[6] The underlying doctrine is that the high temperature of 90° to 94°C bolsters in eliminating the wax and dishwash as detergents are chiefly surfactants. This is due to reduction in the surface tension of water.[6, 9]

Harri's hematoxylin was used to stain the nuclear component of tissue in our study instead of Mayer's hematoxylin, which was used by many researchers and they concluded that the crispness of nuclear staining with Mayer's hematoxylin was merely equivalent to that with Harri's hematoxylin.[1, 3] Firstly, Harri's hematoxylin is more commonly used in the H and E stain. Secondly, it can be used progressively, but is used regressively in our study.[5] As it is used regressively, it disperses the stain rapidly over the entire cell.[7, 8] Differentiation with 1% acid alcohol was done to remove traces of hematin binding to cytoplasm, making it free to bind to the eosin stain. [5] Thirdly, Harri's hematoxylin also contains glacial acetic acid which gives precise nuclear staining.[7, 8]

Tap water was used for bluing in our study instead of lithium carbonate. Hard tap water is alkaline in nature and turns the colour of Hematoxylin blue. Additionally, it is more crude and the colour of the slides is stable.[11] *Ankle MR and Joshi PS* (2011) in their study observed that adequate nuclear staining, better clarity and better crispness of sections was seen in the xylene free group.[1]

Ramulu et al (2012) in their study noted that sections stained with XAF method demonstrated adequate nuclear staining and better clarity of sections compared to the conventional H and E method.[5] *Pandey et al* (2014) observed that the XAF sections scored better in the parameters of nuclear staining, cytoplasmic staining, clarity and crispness of staining.[2] *Anuradha Ananthaneni et al* (2014) noted that XAF sections stained better in terms of uniformity of staining.[9]

Other sections and tissues like melanin pigment [Figure4], adipose tissue [Figure 5], muscle fibers [Figure 6] and nerve fiber bundle [Figure7] were also stained with H and E staining method and XAF H and E staining method which showed that the staining by latter one was better than the conventional one in addition to the above mentioned parameters.

On the basis of scores obtained in both the methods, results depicted that the staining was adequate for diagnosis in 96.66% of group A sections and 100% in group B sections ($Z=1.106$, $P > 0.05$) [Table 2] but the difference obtained was not statistically significant as P value was more than 0.05. This shows that XAF H and E staining was at par with that of the conventional H and E staining method and liquid DWS can be used as a substitute in XAF staining method but still, liquid DWS has not yet replaced xylene because firstly, it is temperature sensitive. Even a slightest fall in temperature leads to improper wax removal from the section whereas sections were lost due to the rise in temperature. Secondly, it can only be used as deparaffinizing and not dehydrating agent because dehydrating agent should be miscible with both wax and alcohol.[9] Thirdly, liquid DWS solution has to be freshly prepared everytime as wax particles remain suspended in the solution.

SCOPE FOR THE STUDY

XAF sections need to be studied after 1 year to see that sections have not faded and the staining quality is not compromised.[1]

CONCLUSION

The present study showed that XAF H and E staining procedure carried out by using a liquid DWS solution is at par with the conventional H and E staining procedure. It produces quality staining with sufficient clarity and a crisp nuclear and cytoplasmic staining. It has added advantages of being non toxic, non hazardous, non inflammable, economical, less staining time and very easy to handle. So, Liquid DWS can be used as a substitute to xylene and alcohol in routine H and E staining procedure.

CONFLICTS OF INTEREST

The authors declare they have no potential conflict of interests regarding this article.

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