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Comparison of Modified Papanicolaou and Dane-Herman Stain with Routine Hematoxylin and Eosin Stain for Demonstration of Keratin in Tissue Sections

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School of Graduate Studies

This is to certify that the thesis prepared by **Moti Sori** entitled "*Comparison of Modified Papanicolaou and Dane-Herman stain with Routine Hematoxylin and Eosin stain for demonstration of keratin in tissue sections*" is submitted in partial fulfillment of the requirements for the degree of Master of Science in Histotechnology complies with the regulations of the University and meets the accepted standards with respect to the originality and quality.

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Abstract

Background: Keratins are intermediate filament forming proteins with specific physicochemical properties produced in any vertebrate epithelia. Having characteristic findings in many epithelial pathologies made them diagnostically important marker. Hematoxylin and Eosin(HE) stain, the routine dye, does not distinguish different eosinophilic components of connective tissue distinctly. On the other hand, special stains like modified Papanicolaou(Mod Pap) and Dane-Herman(D-H) stain can prove to be a simple solution to detect the presence and pattern of keratin histologically.

Objectives: To compare modified Papanicolaou stain and Dane Herman stain with routine Hematoxylin and Eosin Stain for demonstration of keratin and also there basic staining characteristics on paraffin blocks tissue sections.

Methodology: A total of 116 Formalin-Fixed Paraffin-Embedded(FFPE) tissue blocks were taken of known keratin containing tissue which included Squamous Cell Carcinoma(SCC), Squamous Papilloma, Verrucous Carcinoma and Odontogenic Keratocyst. The tissue blocks were retrieved from the archive of Tikur Anbessa Specialized Hospital(TASH), Department of Pathology. Three sections of 4µm thick tissue from each paraffin block were made and stained with HE, Mod PAP and D-H stains. Then the three stains were evaluated for Nuclear stain(NS), Cytoplasmic stain(CS), Uniformity of staining(US), Background stain(BS) and Keratin pattern(KP) by two pathologists. Finally, the third Senior pathologist was used as a tie breaker.

Result: Mod PAP showed agreement with HE by showing Mc Nemar's P value of 0.219 for NS, 0.727 for CS, 0.851 for US, 0.824 for BS and 1.00 for KP. Whereas D-H showed disagreement with HE for the first four parameters(P=0.000). But D-H showed agreement for KP. Thus, these results show that keratin was stained by D-H, Mod PAP and HE stains distinctly. Mod PAP stain showed similar result when compared with HE stain with parameters-NS, CS, US and BS. However, D-H stain showed statistically significant difference when crosschecked with HE stains. Special stains agreement were analyzed using McNemar as a statistical data tool, $P < 0.05$ were considered statistically significant.

Conclusion: Mod PAP and D-H stains showed similar result for staining of keratin compared to HE stain. For the other four parameters Mod PAP showed an excellent result but not D-H.

Key Words: Keratin, Dane-Herman, Modified Papanicolaou, Hematoxylin and Eosin stain

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List of Abbreviations/Acronyms

AA	Acid Alcohol
AAU	Addis Ababa University
AB	Alcian Blue
BS	Background Stain
CS	Cytoplasmic Stain
D-H	Dane-Herman method
DPX	Distyrene Plasticizer xylene
DRERC	Departmental Research Ethics Review Committee
DW	Distilled Water
EA-50	Eosin Azure -50
FFPE	Formalin-Fixed Paraffin-Embedded
HE	Hematoxylin and Eosin stain
ICK	Individual cell keratin
IHC	Immunohistochemistry
KP	Keratin Pattern
Mod PAP	Modified Papanicolaou Stain
NS	Nuclear Stain
OG-6	Orange Green -6
PI	Principal Investigator
SCC	Squamous cell Carcinoma
SPSS	Statistical Package for Scocial Sciences
TASH	Tikur Anbessa Specialized Hospital
TW	Tap Water
US	Uniformity of Staining
VIP	Vacuum Infiltration Processing

Operational Definition

Nuclear stain: the intensity of basophilia in a cell.

Cytoplasm stain: the intensity of eosinophilia in a cell.

Adequacy/Inadequacy of staining: the sufficiency/insufficiency of nuclear or cytoplasmic stain intensity of a given staining found in a cell or tissue.

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

Background stain: the staining of tissue or cell parts by a given stain other than the staining property of that particular stain

E.g. if the nucleus is stained by eosin or if cytoplasm is stained by hematoxylin due to PH fluctuation in the staining process or due to technical error.

Keratin Pattern: the presence of a keratin structures in a tissue section.

Introduction

1.1 Background

Keratins are defined as intermediate filament forming proteins with specific physicochemical properties produced in any vertebrate epithelia [1]. Keratins are one of the major and key structural proteins found at their highest concentration and diversity in the keratinocytes of cutaneous as well as oral epithelium and account for almost 80% of the total protein content in differentiated cells of stratified epithelia [2]. In stratified epithelia, the basal cells are attached to the basement membrane and the most superficial of the suprabasal cell layers form the surface of these epithelia. Only the cell in the basal stratum are mitotically active and replenish the loss of cells on the surface of the superficial stratum. In the intermediate stratum of a stratified epithelium, the cells undergo various processes of differentiation, such as keratinization[3].

Keratinization, also termed as cornification, is a process of cytodifferentiation which the keratinocytes undergo when proceeding from their post germinative state (stratum basale) to finally differentiated, hardened cell filled with protein, constituting a structurally and functionally distinct keratin containing surface layer such as stratum corneum. Squamous cell carcinoma(SCC) is characterized by squamous differentiation (often seen as keratinization, sometimes with keratin pearl formation) and invasive growth with disruption of the basement membrane[4]. So the main function of cytokeratins along with microtubules and microfilaments is to provide with structural integrity and mechanical resiliency to all the eukaryotic cells[5].

A wide range of disorders occur because of mutation in the gene encoding for the various keratin proteins. These disorders comprise of the lesions affecting the skin and mucous membrane depending on the distribution of keratin and certain disorders may present with both skin and oral manifestations[1]. Observing keratin formation in a given tissue section supports diagnosis[6]. For example, The identification and staining of individual cell keratin and keratin pearl is important in histopathological grading and diagnosis of Oral SCC[7,8,9,10]. The quality and quantity of keratin synthesis reflects the differentiation level of normal and abnormal epithelial cells[11,12,13,14].

Mod PAP and D-H are the two special stains that were used in this study beside the routine stain HE. Special stains provide valuable and cost-effective information for the pathology laboratory[15].

Dane and Herman found D-H stain by modifying Kreyberg's method . Their method substituted phloxine B for erythrosin B; orange G for saffron and alcian blue 8GS for alcian green 2GX [16,17]. The first researchers who tried Papanicolaou stain on paraffin block tissue section were Johnson P.L. and Klein M.N in 1956 [18]. Then in 1983, Elzay R.P. modified Papanicolaou staining which is commonly used in exfoliative cytology to stain keratin section by increasing the staining time for the three basic Papanicolaou stains. He also incorporated phloxine B as an additional staining component[19].

In this study, Mod.PAP and D-H stains keratin demonstration capability were crosschecked with that of routine HE stain. Formalin-fixed paraffin-embedded blocks tissue sections were the specimen type used in the study.

1.2. Statement of the Problem

Histological staining has been extensively used in pathology laboratories not only because it is easy to perform, but also it has relatively low economic cost. Moreover, it allows to identify several types of cells and provides invaluable information about cell structures, tissue morphology and architectural changes associated with certain diseases [20].

HE stain has always been considered the gold standard in staining tissues. However, this staining technique has its limitations; for example, the color contrast cannot be appreciated at all times, which leads to ambiguity in diagnosis. Especially, in cases like moderately or poorly differentiated SCC's, it is difficult to identify the epithelial infiltration into the connective tissue and the keratin pearl formation[21].

For diagnosis purpose, it is preferred to have a stained tissue section having sharp contrast in color and good nuclear detail. In histological sections, simultaneous demonstration of prekeratin, keratin and mucin is an efficient and reliable aid in establishing cellular origins of pathological tissues [16]. Unlike HE, Mod PAP and D-H can stain prekeratin, keratin and mucins.[16,19].In routine H-E staining, structures like collagen, muscle, and keratin are eosinophilic i.e. their color is uniform. But Mod PAP and D-H stain them differently. Both special stains can stain keratin and the histological demonstration of keratin is important for diagnosis of SCC. Those stains help in assessing the degree/ pattern of differentiation for SCC[22].

Keratins have been proven to be a useful diagnostic and prognostic marker in epithelial malignancies[23]. Keratins are frequently the most abundant cellular proteins. They constitute the major component of the cytoskeleton of all epithelia. Intermediate filaments provide mechanical support for the cells and nucleus. Keratins have a number of distinct advantages to differentiate epithelial tumors from mesenchymal tumors. Though IHC tests are gold standard for staining keratin, they are not affordable in developing countries like Ethiopia. They are also time consuming and not available in the local market. On the other hand, mod PAP and D-H reagents are cheap, available in the local market and have short staining time.

In conclusion, Mod PAP and D-H enhanced nuclear stain and better cytoplasmic differentiation renders tissue morphology more conspicuous and readily interpreted than with routine HE methods[18]. Furthermore, in establishing a definitive diagnosis, it is advantageous to demonstrate

the degree of keratinization or the presence and / or absence of it by using a stain that clearly and differently stains keratin[22]. This study was intended to check if the special stains mod PAP and D-H stain could stain keratinized cell in a way better than HE stain. So in this study Mod. PAP and D-H stain were compared with routine HE in demonstrating keratin using tissue section from selected cases of oral cavity and skin biopsies.

1.3. Significance of the Study

Squamous cell carcinomas are commonly reported in TASH[24], Histopathology laboratory. Some SCC's are difficult to diagnose with routine HE stain. The presence or absence of keratinization, as well as the morphological features associated with keratinization, can influence a definitive diagnosis. Though IHC tests are known for their specificity, they are not affordable in developing countries like Ethiopia. The routine HE stain does not distinguish different eosinophilic components of connective tissue, which makes diagnosis difficult. On the other hand, the special stain Mod PAP and D-H stains differentiate eosinophilic components of tissue clearly. Additionally, they distinctly stain prekeratin, keratin and mucin. So doing this research in our lab set up will let us see other options we have for diagnosing keratin disorders. Therefore, this study was intended to compare Mod PAP and D-H with routine HE in aspects of basic staining characteristics and their ability to identify keratin.

2. Literature Review

Johnson P.L. and Klein M.N, conducted a study in New York University, USA on the application of the Papanicolaou stain to paraffin embedded sections in 1956. Tissues were fixed in fixatives like formalin, Carnoy's and Bouin's and this stain were applied on various keratinizing epithelial tissues including hairless skin, hairy skin, feathers, nails, claws, hoof and well-cornified oral mucosa. Johnson P.L. and Klein M.N study showed that Papanicolaou has a better morphology than HE staining. They found that the color pattern was similar in all tissue blocks and various tissue elements showed consistent affinity for a specific component of the stain[18].

Similar study done in Los Angeles, USA by Dane E.T. and Herman D.L in 1963 reported to use D-H stain for demonstrating keratin in paraffin block tissue section. Tissue specimens were fixed in 10% formalin at least 48hrs, embedded in paraffin, cut sections 5-7µm and attached to slide with egg albumen. They reported that acid mucopolysaccharide were stained turquois blue; prekeratin and keratin were orange to red orange [16].

Study was done by Rao RS et al.,in India in 2014; their study compared special stains like Papanicolaou stain and Dane-Herman stain to demonstrate keratin in tissue sections. A total of 80 cases of known pathology for keratin were retrieved from the department archive. Three section of 4µm each from the paraffin blocks were made, stained with HE and the special stains and these were evaluated by 2 pathologists based on the modified scoring criteria from Rahma Al-Maaini and Philip Bryant 2008. The results were tabulated using Chi-square and kappa statistics. The statistical values for identification of the type of keratinization was insignificant showing that ortho and parakeratinized epithelia could be correctly identified by both HE as well as special stains. Furthermore, all the special stains showed a positive result and statistical significance($P<0.001$) with respect to the staining of keratin. The study concluded that Mod PAP and D-H stains distinctly stained the keratin with a higher intensity, HE proves to be overall better stain with respect to specificity[25].

Preethi S. and Sivapathasundharam B. conducted a research, in Tamil Nadu, India in 2015. They compared hematoxylin and eosin stained tissue sections with Papanicolaou stained tissue sections. A total number of 70 paraffin block embedded tissue sections were taken and tissue sections of 4–5 µm were made from each block and these tissues were then stained using routine HE and

modified PAP techniques. The results of the stained slides revealed a marked difference in staining with respect to color balance, contrast and intensity between HE and PAP stains. Areas of keratinization were stained from shades of orange to pink depending on the degree of keratinization. In moderately differentiated squamous cell carcinoma, areas of keratin pearl formation and individual cell keratinzation were clearly demonstrated; these were not apparent in the HE stained slides. This study suggest that the PAP stain more clearly demonstrated key morphological features, associated with keratinization, and it may be considered as a supplemental staining procedure, aiding in diagnosis[21].

Study was done in Tamilnadu India by Caliaperoumal S.K.,in 2017 , to evaluate and compare the distinct staining and identification of Keratin pearl and Individual cell keratin(ICK) by routine Hematoxylin and Eosin stain, Modified Mallory's and Modified Papanicolaou Stain. A total number of 38 paraffin embedded tissues of known cases of well-differentiated and moderately-differentiated SCC were taken and 3 sections of 4-5 micron thickness from each block were cut and stained with HE stain, Modified Mallory's and modified Papanicolou stain. The Keratin pearl and ICK were distinctly and clearly stained by modified Papanicolou, modified Mallory's stain compared to HE stain. The positive staining of Keratin pearl and ICK by modified Papanicolou stain was statistically significant than modified Mallory's and HE stain at P=0.033 and P=0.001[26]. Another study done in Puducherry, India by Caliaperoumal S.K.,in 2016 on the comparison of modified papanicolaou and hematoxylin and eosin stain in demonstration of keratin pearl and individual cell keratin in oral squamous cell carcinoma proved the same thing[27].

Histochemical study was done by Mumtaz W.R., et al.in Karnataka, India in 2016, on Micrometastasis Detection in Nodal Tissues. The result showed Papanicolaou stain was the only stain useful in detecting micrometastasis which accounted to 7% of non metastatic lymph node sections used in the study. They concluded that Papanicolaou stain has definitely proved valuable in detecting micrometastasis over routine Haematoxylin and Eosin with an added advantage of being economical, easily available and technique insensitive[28].

Another study was conducted by Srivastava A. et.al., in Uttar Pradesh, India in 2017 on the comparison of modified Kreyberg's, modified Papanicolaou, Ayoub-Shklar, and Haematoxylin and Eosin stains to demonstrate keratin in paraffin embedded tissue sections. A total of 75 paraffin embedded tissue blocks were taken of known keratin containing tissue. which included Well

Differentiated Squamous Cell Carcinoma, Hyperkeratosis with or without dysplasia, Orthokeratinized Odontogenic Keratocyst and Verrucous Carcinoma. Four sections measuring 4µm in thickness of each block were cut and were stained with all the mentioned stains. The result showed that Keratin was stained by all four stains distinctly in Well Differentiated Squamous Cell Carcinoma, Hyperkeratosis with or without dysplasia, Orthokeratinized Odontogenic Keratocyst and Verrucous Carcinoma. Based on the overall staining intensity and pattern the study concluded that all four stains can demonstrate keratin. Ayoub-Shklar and HE stains can be used efficiently to stain keratin but have certain limitations[29].

Another study done by Caliaperoumal S.K., in Puducherry, India in 2017, evaluated and compared the distinct staining and surface keratin in verrucous carcinoma and normal keratinizing epithelium by routine hematoxylin and eosin stain, modified papanicolou stain and modified Mallory's stain. The known cases of verrucous carcinoma and tissues from normal keratinizing epithelial regions with sufficient paraffin embedded tissue were selected. The three sections from each block were stained with HE stain, modified Papanicolou stain and modified Mallory's stain. The positive staining of surface keratin was significantly associated at p=0.001 in verrucous carcinoma and normal epithelium with all stains. However, the positivity was more associated with modified Papanicolou stain followed by modified Mallory stain least by heamatoxylin and eosin Stain[30].

Given the finding in the reviewed articles, it is very important to compare Mod PAP and D-H stain with routine HE in our laboratory set up so that Pathologists could get options in some difficult cases of Keratin disorder where difficulty is faced in HE stain.

3. Objectives

3.1. General Objective:

To compare modified Papanicolaou stain and Dane Herman stain with routine Hematoxylin and Eosin Stain for demonstration of keratin and also there basic staining characteristics on paraffin blocks tissue sections.

3.2. Specific Objectives:

(i) To compare Mod PAP stain and D-H stain with routine HE stain in identification of keratin present in the tissue section.

(ii) To determine nuclear staining and compare Mod PAP and D-H stained slides with routine HE stained slides.

(iii) To elucidate the difference on cytoplasmic staining between Mod PAP and D-H stained tissue section and compare them with HE stained tissue sections.

(iv) To compare uniformity of stained tissue sections of Mod PAP stain and D-H stain with routine HE stain.

(v) To compare background staining of Mod PAP stain and D-H stain slides with routine HE stain.

4. Materials and Methods

4.1. Study Area

Tikur Anbessa Specialized Hospital(TASH) is found in the capital city of Ethiopia. TASH is Ethiopia's largest general public hospital and one of University Hospitals in the country. It is located in Addis Ababa at Lideta sub-city opposite to Immigration office of Ethiopia. The hospital provides a tertiary level referral treatment and is open 24 hours for emergency services. The hospital is administered by Addis Ababa University and is one of the oldest teaching hospitals. It provides teaching for about 300 medical students and 350 residents every year. It is currently the main hospital in Ethiopia that provides an oncology service.

This study was conducted in the Histopathology laboratory of TASH. The laboratory receives about 10,000 biopsy specimens annually.

4.2. Study Design and Period

This is a cross sectional study and the study period was from April, 2018- March, 2019.

4.3. Sample

4.3.1. Source Sample:

All biopsies sent to TASH, Histopathology Laboratory in the year 2017.

4.3.2. Study Sample:

Oral cavity and Skin biopsies sent to TASH, Histopathology Laboratory in the year 2017. FFPE tissue blocks from oral cavity and skin biopsies reported of having keratin disorder which included SCC, Squamous Papilloma, Verrucous Carcinoma or Odontogenic Keratocyst were used.

4.4. Sample size and Sampling methods

A total of 116 FFPE tissue blocks from oral cavity and skin biopsies reported of having keratin disorder during the study period were included and convenient type of sampling method was used.

4.5. Eligibility

4.5.1. Inclusion Criteria

Oral and skin biopsies that came to TASH, Histopathology laboratory in 2017 and were reported as SCC, Squamous Papilloma, Verrucous Carcinoma or Odontogenic Keratocyst were included.

4.5.2. Exclusion Criteria

Oral and skin biopsies that came to TASH, Histopathology laboratory in 2017 and were reported as SCC, Squamous Papilloma, Verrucous Carcinoma or Odontogenic Keratocyst but those that had very small tissue left in their blocks(< 5mm) were excluded.

4.6. Variables

4.6.1. Dependent Variables

- Keratin patterns
- Nuclear detail
- Cytoplasmic transparency
- Uniformity of staining.
- Background stain

4.6.2. Independent Variables

- Routine Hematoxylin and Eosin
- Dane-Herman stain
- Modified Papanicolaou stain

4.7. Study procedures and Data Collection

4.7.1. Biopsy specimen preparation

A. Fixation: is the first step in the process of biopsy specimen preparation. Fixation, or protein stabilization, preserves the specimen in a reproducible manner that most closely resembles its living state. Tissue biopsies used in this study were taken from tongue, buccal area, lip, skin, jaw, gingiva, mandible and hard palates. Those tissues were fixed in 10% formalin solution.

B. Tissue processing and Embedding

Tissue processing is a process of passing biopsy in four steps (fixation, dehydration, clearing and infiltration) to allow sectioning of specimen (please refer to the appendix A for the tissue processing procedures). All blocks that were used in this study were processed in tissue tek-II V.I.P 150 tissue processor. Then the tissue were externally supported or embedded in paraffin wax.

C. Microtomy(Tissue Cutting): It is a process of producing thin slices (3-5 µm) of the specimen. We used a functional rotary microtome for sectioning. This is a final step in preparing the specimen for staining. These thin sections were placed onto glass slides, dried and then stained.

4.7.2. Staining

Mod pap, D-H and HE stains were the three stains used in this study. Common steps shared by those stains were deparaffinization, dexylenization, rehydration and after staining dehydration, clearing and coverslipping.

4.7.2.1. Reagents

a. HE stain

- Harri's hematoxylin
- 0.3% Eosin Y
- 0.3% Acid alcohol
- 0.5% Lithium carbonate

b.D-H stain

- Mayer's Hematoxylin
- 1% Phloxin B
- 0.5 % Alcian Blue
- Orange G-6

c.Mod PAP stain

- Harri's hematoxylin
- 1% Phloxin B
- Orange G-6
- Eosin-Azur 50
- 0.3% Acid alcohol
- 0.5% Lithium carbonate

4.7.2.2. Solution Preparation

Please refer to the appendix B for the detail solution preparation procedures.

4.7.2.3. Procedure

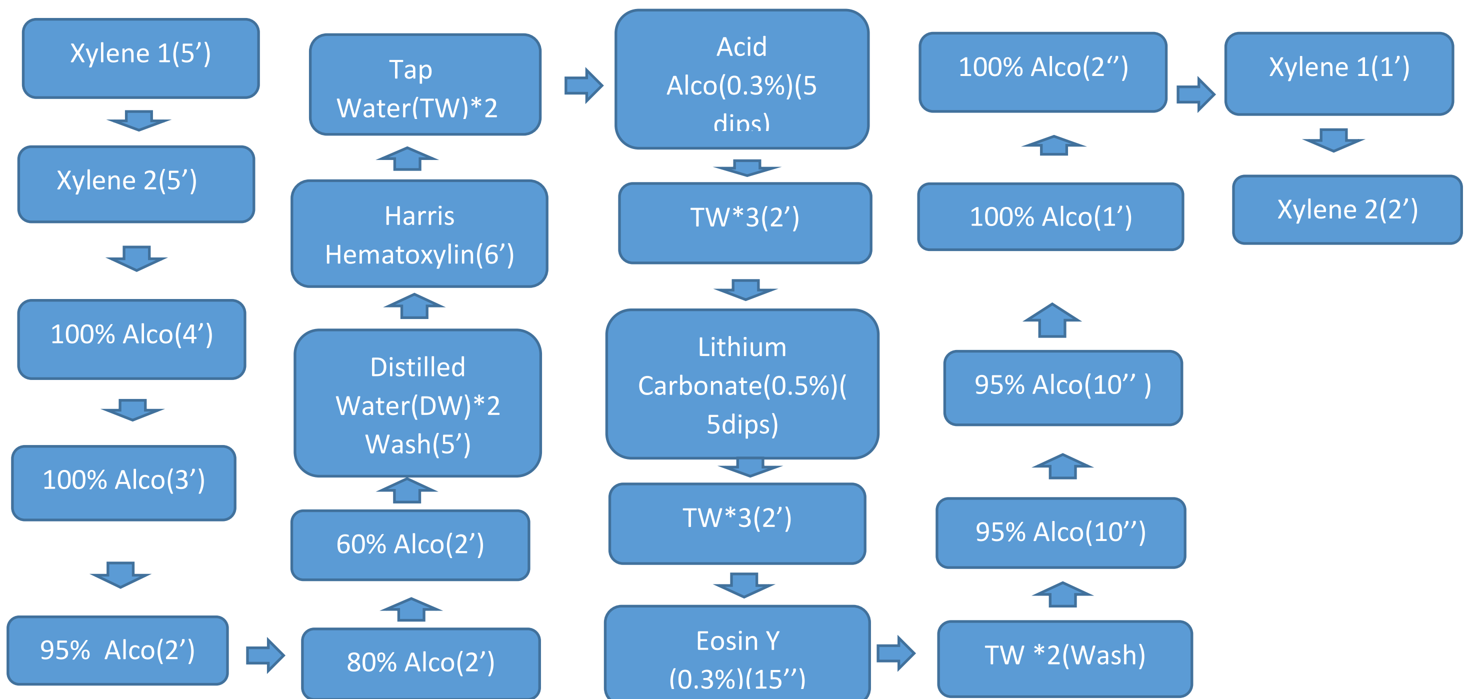

Figure 1. Hematoxylin and Eosin staining procedure

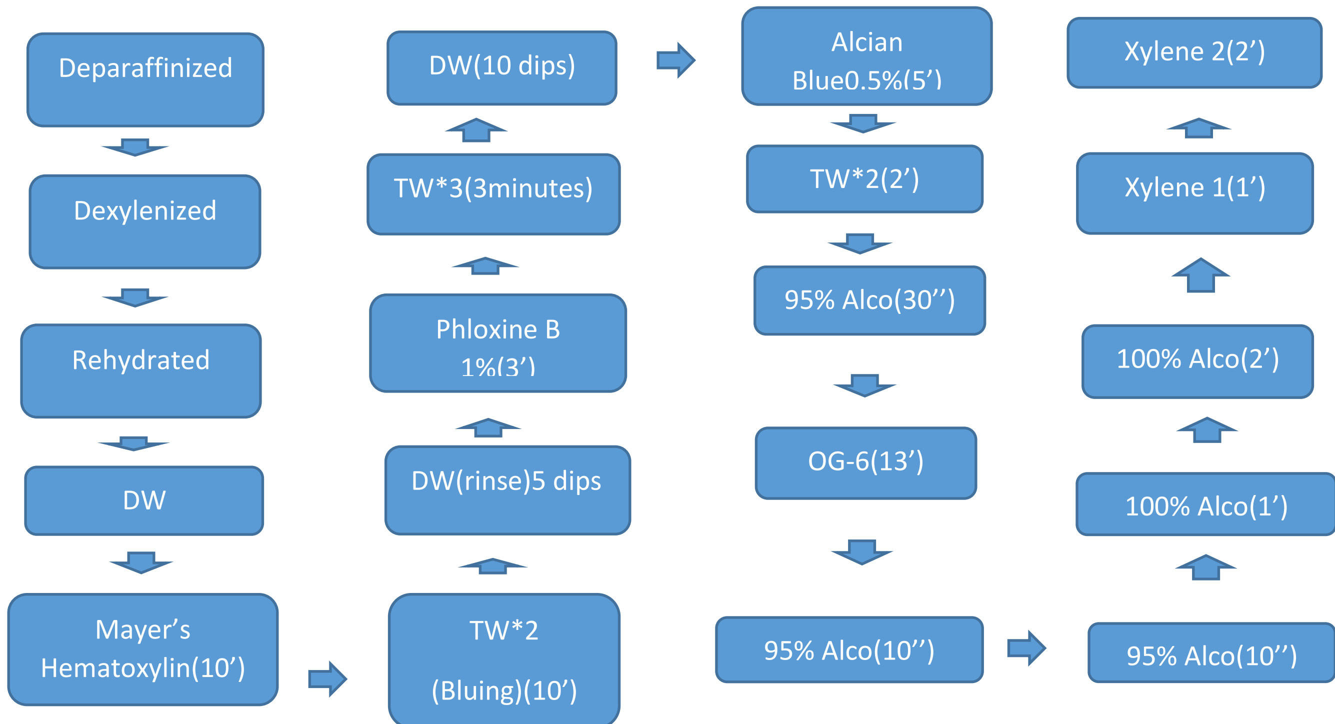

Figure 2. Dane-Herman staining procedure

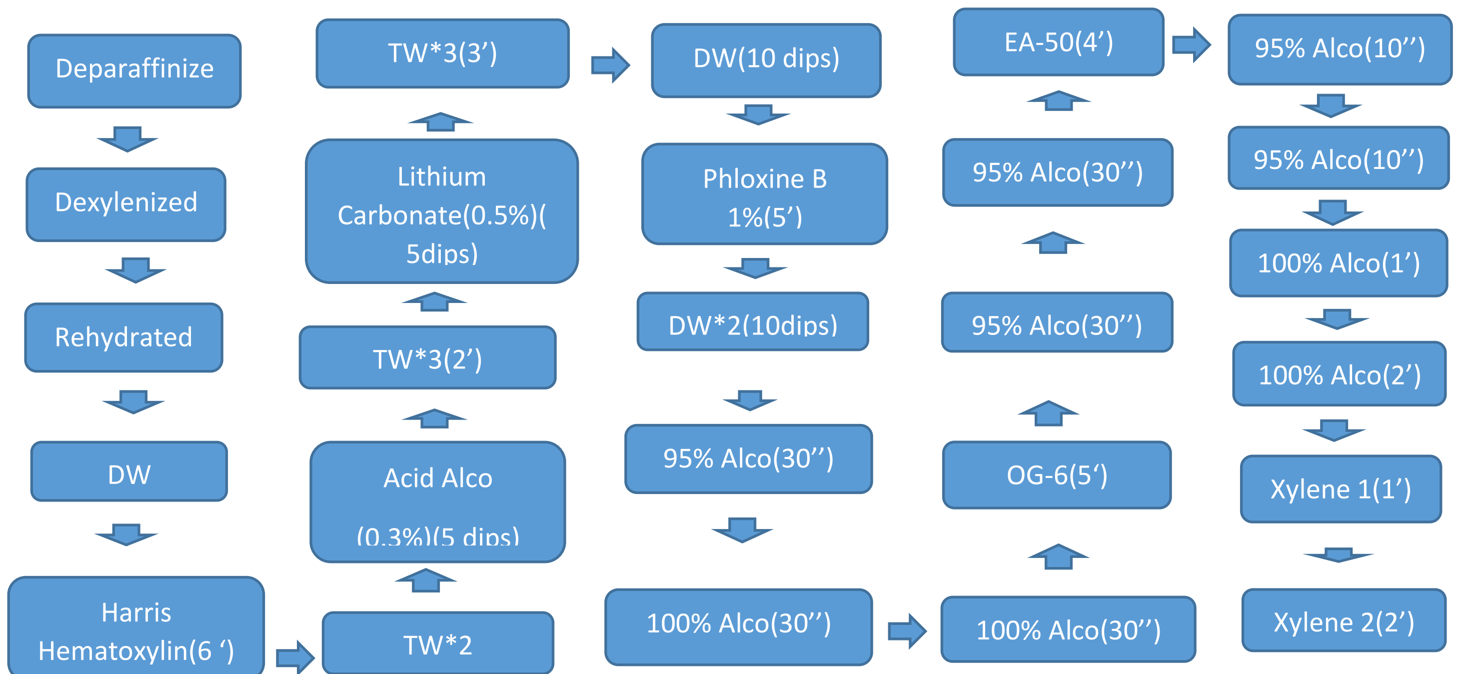

Figure 3. Modified Papanicolaou staining procedure

4.7.2.4. Drying and Coverslipping

The drying of stained tissue sections took place using two containers of 95 and 100% ethanol. Then slides were cleared in two containers of xylene. After that, each slide was coverslipped using a mountant called Distyrene Plasticizer xylene(DPX).

4.7.2.5. Staining Results

a.HE stain	b.D-H stain	c.Mod PAP
•Nuclei...........Blue-Violet •Cytoplasm... Orange-red •Keratinized cell ..Orange-red •Keratin pearl in SCC..Pink	•Nuclei....Blue-violet •Cytoplasm...eosinophilic •Keratinized cell..eosinophilic •Keratin pearl in SCC..orange-red	•Nuclei....Blue •Cytoplasm...pink •Keratinized cell...Orange •Keratin pearl in SCC.orange-pink

4.7.3. Assessment of Stained tissue sections

All 116 blocks stained in triplicate slides were evaluated by two pathologists independently. To avoid bias during evaluation, the two pathologists were blinded i.e. the three stained slides were given in a coded form. Then the tiebreaker, the senior pathologist, reported the final results.

Mod PAP, D-H and HE stained sections were graded based on the following five parameters:

- Nuclear staining (adequate = score 1, inadequate = score 0)
- Cytoplasmic staining (adequate = score 1, inadequate = score 0)
- Uniformity of staining (present = score 1, absent = score 0)
- Background staining (present = score 1, absent = score 0)
- Keratin pattern (positive = score 1, negative = score 0).

A score of 0/1 were given to each of these parameters and McNemar's test were used to compare the 116 matched pairs of D-H and HE stains and 116 matched pairs of Mod PAP and HE stains. In this study HE stain was used as standard stain, $p < 0.05$ considered as significant.

4.7.4. Pilot Studies

Five pilot studies were done before the actual study slides were stained by Mod. PAP, HE and D-H stains. For the first and second pilot 4 blocks were used for each trial and for the third, fourth and fifth pilot studies 5 blocks were used for each studies. In each pilot, tonsillar and skin tissues were used us a control. On the first pilot, 1% acid alcohol(AA) were used for differentiation in HE and Mod PAP stains. Seven dips were used for differentiation. The three stains were pale in both nuclear and cytoplasmic parameters and also precipitation like artifacts were observed. Before the second pilot study, each staining solution of HE, D-H and Mod PAP stains were filtered to reduce artifacts. washing time was increased on the second pilot study to avoid staining solutions cross contamination and 0.5% AA were used for the second and third pilot studies but dipping count was reduced from 7 to 5. Improvement were seen in HE, D-H and Mod PAP stains in the aspects of nuclear and cytoplasmic stains. In addition, background stains were reduced and uniformity of staining were improved. On the fourth pilot study, 0.3% AA with 5 dips were used for differentiation. The results of stained slides were very good in all five parameters. Before the fifth pilot study, staining solutions for the three stains including reagents for deparaffinization, dexylenization and rehydration were properly changed. Stained slides showed very good result on the fifth pilot study. The nuclear and cytoplasmic stains were very good, slides showed no background stain and throughout the tissue section stains were Uniform. After the fifth pilot study, the study slides were stained, evaluated and the study data were collected and analyzed.

Finally, to see the possible causes of poor nuclear and cytoplasmic stain of D-H stain seen in some tissue of the study bocks, the six pilot study were done. From tissue blocks that were affected by D-H stain, 10 blocks were randomly selected. Tissue blocks were considered affected if there result were affected in parameters ≥ 3 out of 5 parameters used in the study. So, hematoxylin solution were prepared in 1,1.2,1.3 and1.4gm/l. Ten blocks were grouped in to five and two blocks were used in each group. Hematoxylin staining time were increased to 15 minutes and agitation were avoided in Alcian Blue(AB) solution for the four groups. On the fifth group, the hematoxylin

concentration were 1.4gm/l with staining time 15 minutes and agitation were included in AB solution. The above five groups were run concurrently. Slides showed improvement in nuclear and cytoplasmic stains. This relative change was seen in a group agitation was avoided in AB solution and hematoxylin concentration was 1.4gm/l.

4.8. Data Quality Assurance

The data quality assurance could be grouped in to three phases: pre-analytic, analytic and post-analytic.

4.8.1. Pre-analytic Phase

In this study, FFPE tissue blocks were properly identified by checking type of tissue on the reception room log book and also the diagnosed report. On the report, microscopic description, diagnosis and keratin content were checked.

4.8.2. Analytic Phase

The study tissue blocks were taken from 2017 block archive. Those tissue blocks were processed by one tissue processor i.e. VIP-150 Tissue Tek II. All selected tissue blocks were properly re-embedded. Then, using a properly functioning rotary microtome, water bath and cool plate, tissue blocks were cut-sectioned and mounted on slides. For each block, three tissue sections were taken, code were given for three staining and slides were carefully labeled. Before the staining was undertaken, the expiry date of reagents and staining powders were checked. Using standard protocols, staining solution were prepared. Control tissue were used for nuclear, cytoplasmic and Keratin staining. Tonsillar tissue was used for nuclear staining control, skin was used for Keratin and cytoplasmic staining control[31].

During the staining of study samples i.e. 116 tissue blocks, three experienced laboratory professionals were able to manage HE, Mod.PAP and DH staining using three functional timer. In each batch, control tissue slides were run together. Finally, cover slipping of stained slides were properly done.

Using a well prepared scoring checklist of having five parameters, each slide was examined under olympus microscope by three pathologists. The third pathologist was used as a tiebreaker.

4.8.3. Post-analytic Phase

In this study, complete report of the pathologist was properly decoded and entered to SPSS (Statistical Package for Scocial Sciences) version 21 by the PI.

4.9. Statistical Analysis

The data reports of the tiebreaker entered in to SPSS Version 21. Comparison of matched pairs of stained tissue section slides of D-H and HE, and Mod PAP and HE stains were made separately by using McNemar’s test to show the equivalence/agreement, $p<0.05$ considered as statistically significant.

4.10. Ethical Consideration

The study was carried out after obtaining ethical clearance from Addis Ababa University, College of Health Sciences, Department of Research Ethics Review Committee (DRERC) of the department of pathology. The data from the study sample were used for the purpose of this research and each participant reported result were identified by numerical code. Therefore, anonymity and confidentiality were strictly maintained.

5. Result

5.1. Evaluation Characterization of D-H, Mod PAP and HE stained tissue sections

For the demonstration of keratin in FFPE tissue sections, three stains were used i.e. HE, Mod.PAP and D-H stains. A total of 116 paraffin blocks were included and from each block three tissue sections were taken. All 348 (116 blocks*3) slides were evaluated by two anatomical pathologists independently and the third senior pathologist was used as a tiebreaker. All pathologists used a checklist that contains five parameters. Those are nuclear staining, cytoplasmic staining, uniformity of staining, background staining and keratin pattern.

5.2. Overall frequency of the three stains

The result showed 74 D-H, 111 Mod PAP and 115 HE stained sections were adequate while 42 D-H, 5 Mod PAP and 1 HE stained sections were inadequate for nuclear staining respectively. Cytoplasmic staining was reported adequate for 65 D-H, 113 Mod PAP and 111HE and inadequate for 51 D-H, 3 Mod PAP and 5 HE stained sections respectively. The pathologist reported 44 D-H, 88 Mod PAP and 86 HE stained sections were present and 72 D-H, 28 Mod PAP and 30 HE stained sections were absent for uniformity of staining respectively. Background staining was reported present for 66 D-H, 10 Mod PAP and 12 HE and absent for 50 D-H, 106 Mod PAP and 104 HE stained sections respectively. Keratin pattern of staining was reported positive for 83 D-H, 83 Mod PAP and 83 HE and negative for 33 D-H, 33 Mod PAP and 33 HE stained sections respectively.

This research report showed the overall frequency of the five parameter for D-H, Mod.PAP and HE staining as follows(Table 1).

Table 1: Overall figure of the frequency of the parameters with the respective staining methods

Parameters		**D.H**	**Modified pap**	**H & E**
Nuclear staining	Inadequate	42(36.2%)	5(4.3%)	1(0.9%)
	Adequate	74(63.8%)	111(95.7%)	115(99.1%)
Cytoplasmic staining	Inadequate	51(44%)	3(2.6%)	5(4.3%)
	Adequate	65(56%)	113(97.4%)	111(95.7%)
Uniformity	Absent	72(62.1%)	28(24.1%)	30(25.9%)
	Present	44(37.9%)	88(75.9%)	86(74.1%)
Background staining	Absent	50(43.1%)	106(91.4%)	104(89.7%)
	Present	66(56.9%)	10(8.6%)	12(10.3%)
Ketatin parameter	Negative	33(28.4%)	33(28.4%)	33(28.4%)
	Positive	83(71.6%)	83(71.6%)	83(71.6%)

5.3. Nuclear Staining

Nuclear Staining was reported inadequate/adequate depending on the ability to show or stain different parts of nucleus and their division clearly. When the matched pair slides of the two stains are compared for one parameter and score similar result(Inadequate/Adequate), it was reported as agreement. But for differ score results, it was reported as disagreement.

On nuclear parameter this study compared D-H and HE stains and the result showed agreement in 75 matched pairs. Where as, disagreement occurred in 41 pairs. All 41 mismatched pairs were in the advantage of HE stain. The McNemar p value was 0.000. On the other hand, Mod PAP and HE stains were compared and 110 matched pairs agreed. From the 6 mismatched pairs, 5 of them were in favor of HE stain. The McNemar p value was 0.219(Table 2).

Table 2: Comparing Nuclear staining of the Hematoxylin and Eosin stained sections with Dane-Herman and Modified Papanicolaou stained sections

Pathologist III		**D.H**				**Modified pap**			
		Inadequate	Adequate	Total	McNemar's *p* value	Inadequate	Adequate	Total	McNemar's *p* value
HE	Inadequate	1	0	1	P=0.000	0	1	1	P=0.219
	Adequate	41	74	115		5	110	115	
	Total	42	74	116		5	111	116	

Figure 4: Photomicrography of stained histological section of Squamous cell carcinoma showing adequate Nuclear Staining; (A) Hematoxylin and Eosin stain 40X, (B) Dane-Herman stain 40X and (C) Modified Papanicolaou stain 40X.

5.4. Cytoplasmic Stain

Cytoplasmic stain was reported inadequate/adequate depending on the ability to show or stain cytoplasm clearly or separately.

On this study, D-H and HE stains were compared for Cytoplasm parameter and there results agreed in 66 matched pairs and disagreed in 50 pairs. Out of 50 mismatched pairs, 48 pairs were in favor HE stain. The McNemar p value was 0.000. On other hand, ModPAP and HE stains agreed in 108 matched pairs and disagreed in 8 pairs. Out of 8 mismatched pairs, 5 pairs were in favor of Mod PAP stain. The McNemar p value was 0.727(Table 3).

Table 3: Comparing Cytoplasmic staining of the Hematoxylin and Eosin stained sections with Dane-Herman and Modified Papanicolaou stained sections

Pathologist III		**D.H**				**Modified pap**			
		Inadequate	Adequate	Total	McNemar's *p* value	Inadequate	Adequate	Total	McNemar's *p* value
HE	Inadequate	3	2	5	P=0.000	0	5	5	P=0.727
	Adequate	48	63	111		3	108	111	
	Total	51	65	116		3	113	116	

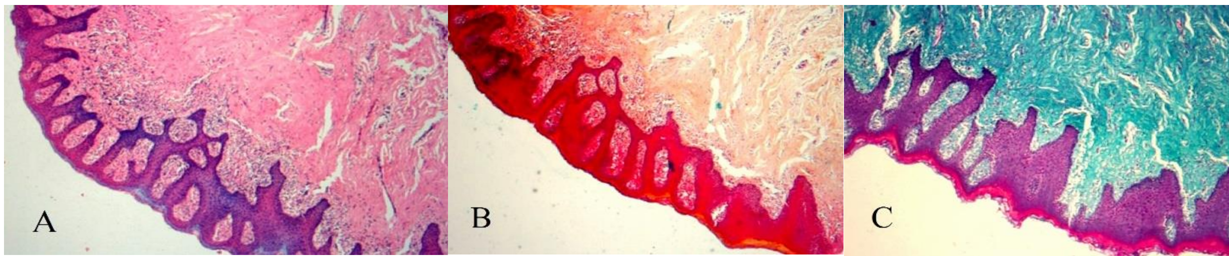

Figure 5: Photomicrography of stained histological section of Squamous Papilloma showing adequate Cytoplasmic Staining; (A) Hematoxylin and Eosin stain 4X , (B) Dane-Herman stain 4X and (C) Modified Papanicolaou stain 4X

5.5. Uniformity of Staining

Uniformity of staining presence/absence was reported based on sameness of stain throughout individual cell or tissue section.

D-H and HE stains were compared for Uniformity of staining and the result showed agreement in 42 matched pairs. In 74 pairs disagreement occurred. Out of 74 mismatched pairs, 58 pairs favored HE stain. The McNemar p value was 0.000. On the other hand, Mod PAP and HE stains were compared and agreement occurred in 88 matched pairs. From 28 mismatched pairs, 15 pairs favored Mod PAP and 13 pairs favored HE stain. The McNemar p value was 0.851(Table 4).

Table 4: Comparing Uniformity of the Hematoxylin and Eosin stained sections with Dane-Herman and Modified Papanicolaou stained sections

Pathologist III		**D.H**				**Modified pap**			
		Absence	Presence	Total	McNemar's *p* value	Absence	Presence	Total	McNemar's *p* value
HE	Absence	14	16	30	P=0.000	15	15	30	P=0.851
	Presence	58	28	86		13	73	86	
	Total	72	44	116		28	88	116	

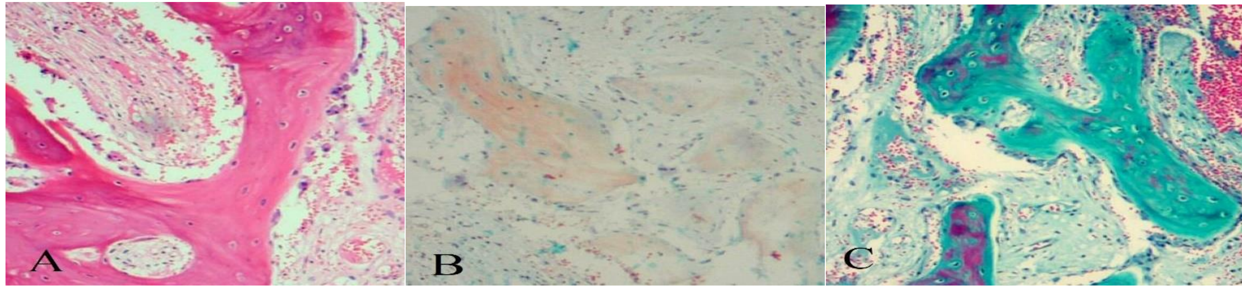

Figure 6: Photomicrography of stained histological section of Odontogenic Keratocyst showing Uniformity of Staining; (A) Hematoxylin and Eosin stain 10X, (B) Dane-Herman stain 10X and (C) Modified Papanicolaou stain 10X

5.6. Background Stain

Background stain(BS) presence/absence was reported depending on the obscuring of any tissue part by stain other than the tissue nature. BS is absent if there is no background stain or unusual stain on nucleus, cytoplasm or keratin part of tissue section and BS is present if there is background stain or unusual stain on nucleus, cytoplasm or keratin part of tissue section.

D-H and HE stains were compared for background staining and the result showed agreement in 60 matched pairs. From 56 mismatched pairs, 55 pairs favored HE stain. The McNemar p value was 0.000. On the other hand, Mod PAP and HE stains were compared and agreement occurred in 96 matched pairs. Out of 20 mismatched pairs, 9 pairs favored HE and 11 pairs favored Mod PAP. There McNemar p value was 0.824(Table 5).

Table 5: Comparing background staining of the Hematoxylin and Eosin stained sections with Dane-Herman and Modified Papanicolaou stained sections

Pathologist III		**D.H**				**Modified pap**			
		Presence	Absence	Total	McNemar's *p* value	Presence	Absence	Total	McNemar's *p* value
HE	Presence	11	1	12	P=0.000	1	11	12	P=0.824
	Absence	55	49	104		9	95	104	
	Total	66	50	116		10	106	116	

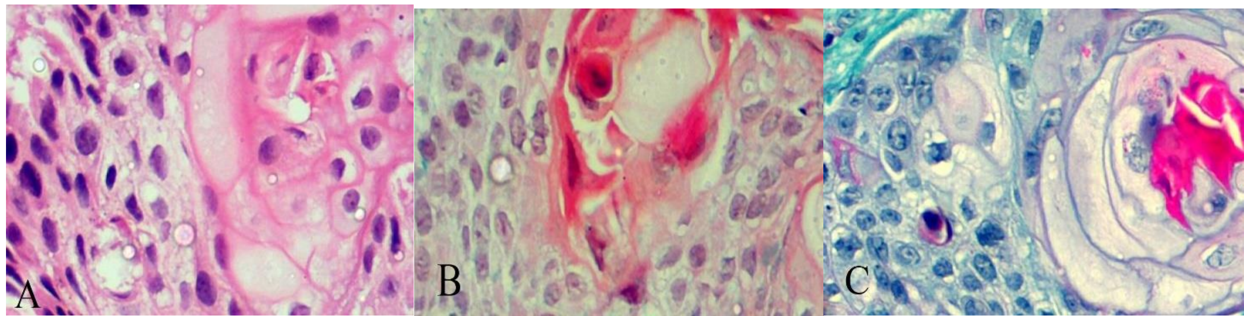

Figure 7: Photomicrography of stained histological section of Squamous cell carcinoma showing no Background Staining; (A) Hematoxylin and Eosin stain 40X, (B) Dane-Herman stain 40X and (C) Modified Papanicolaou stain 40X

5.7. Keratin pattern of staining

Keratin pattern(KP) score 0(negative) if keratin structure of tissue section is not stained orange-red. KP score 1(positive) if keratin structure of tissue section is stained orange- red.

D-H and HE stains were compared for keratin pattern and the result showed agreement in 114 matched pairs(82 pairs were reported positive and 32 pairs were reported negative). In 2 pairs discrepancy occurred. The McNemar p value was 1. This shows strong agreement between the two stains, as P value of McNemar's test is one. Similarly, Mod PAP and HE stains were compared and the result showed agreement in all 116 matched pairs. The McNemar;s P value was 1(Table 6).

Table 6: Comparing keratin pattern of the Hematoxylin and Eosin stained sections with Dane-Herman and Modified Papanicolaou stained sections

Pathologist III		**D.H**				**Modified pap**			
		Negative	Positive	Total	McNemar's *p* value	Negative	Positive	Total	McNemar's *p* value
HE	Negative	32	1	33	P=1	33	0	33	P=1
	Positive	1	82	83		0	83	83	
	Total	33	83	116		33	83	116	

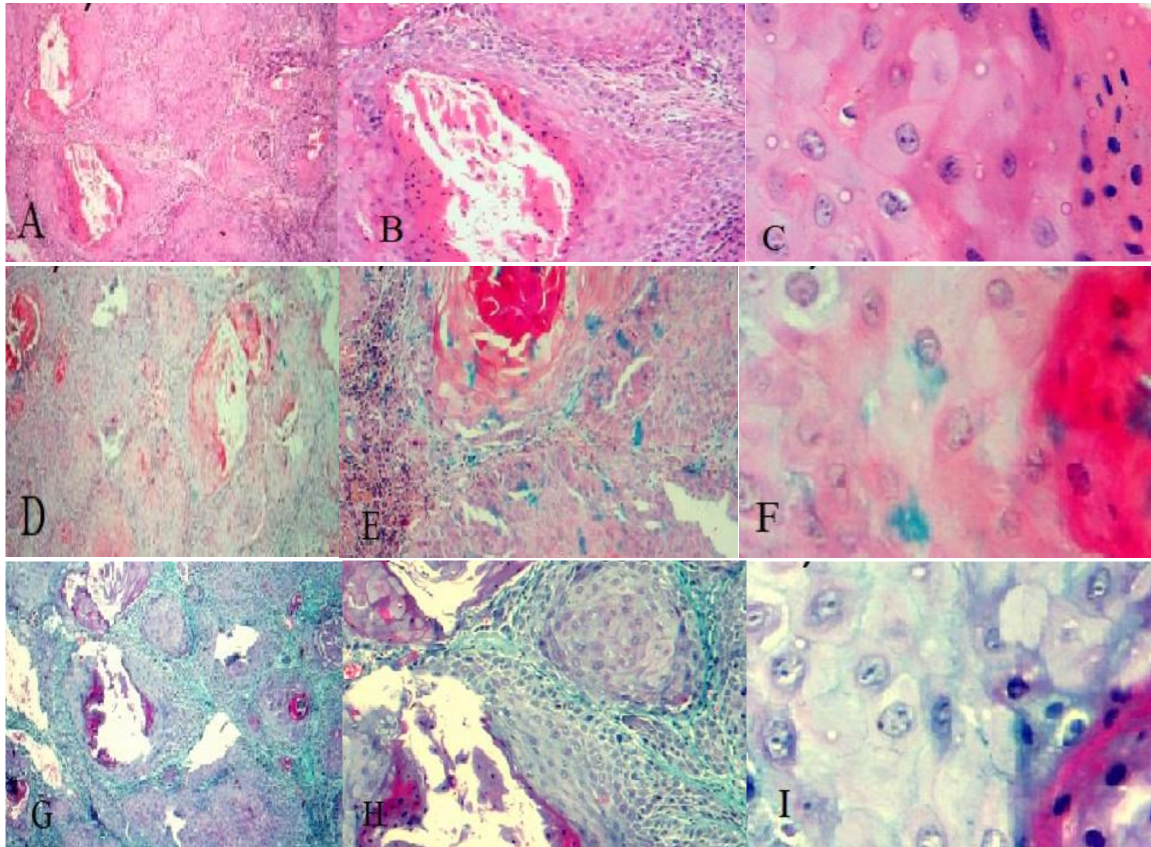

Figure 8: Photomicrography of stained histological section of Squamous cell carcinoma showing Keratinized cells and Keratin Pearls; (A),(B) and (C) Hematoxylin and Eosin stain, (D),(E) and (F) Dane-Herman stain and (G),(H) and (I) Modified Papanicolaou stain.

Magnification of A, D and G:4X, B,E and H:10X and C,F and I:40X

6. Discussion

6.1. The three stains and Parameters used

Hematoxylin and Eosin(HE), Dane – Herman(D-H) and Modified Papanicolaou(Mod.PAP) stain were the three stains used in this study. Routine HE staining was used as a control for evaluating D-H and Mod PAP stains separately for parameters like Keratin Pattern and basic stain characteristics (Nuclear, Cytoplasm, Uniformity and Background staining).

6.2. Keratin Pattern

This study showed that Mod. PAP, D-H and HE staining were excellent at staining Keratin(refer to Figure 8) [19,25,27]. This result opposed with the hypothesis of the study and a research done in India in 2014 supported this result[25]. On the other hand, studies done by Caliaperoumal S.K. in 2017, showed that Mod.PAP is better than HE staining in demonstration of keratin pearl and individual cell keratin (ICK) [26,27].

6.3. Nuclear and Cytoplasmic Staining

D-H stain and HE were compared in this study for both parameter and the result showed that D-H has statistically significant difference with HE stain. The possible cause for poor nuclear and cytoplasm stain may be the acetic acid found in 0.5 % Alcian Blue(AB) solution that is found in D-H staining protocol. A guideline for HE staining says sources of poor nuclear staining could be excessive differentiation time [6,20,32,]. In DH protocol, though AB was used as a counter stain, the acetic acid found in AB solution would get a chance for decolorization of nuclear stain because of study slides spent 5 minutes in AB solution. The other possible cause for poor cytoplasmic staining of D-H could be the absence of general cytoplasmic staining in the protocol[32]. D-H result was normal on the first five pilot studies, this could be due to small sample size used. After the study sample tissue sections were stained or after the study data were collected this gap was observed. Then to look for the possible causes of D-H stain, the final pilot study was done. Changes were seen on final pilot, the nuclear and cytoplasmic staining of D-H stain showed improvement after mayer's hematoxylin concentration of hematoxylin was increased from1gm/l to 1.4gm/l, staining time was increased from 10 to 15 minutes and after agitation was excluded in AB solution.

On the other hand, when Mod PAP were cross checked with HE stain for nuclear stain and Cytoplasmic parameters and this study showed that there is no statistically significant difference between Mod PAP and HE stains. Figures 4 and 5 are good proves to the above statement. However, this result contradicts with the study conducted by Johnson and Klein in 1956 [18].

6.4. Uniformity and Background stain

The result of this study showed that there is a statistically significant difference between D-H and HE stain. On these two parameters, I recommend further studies.

On the other hand, this study also showed that agreement between Mod PAP and HE for both parameters.

In the present study, Mod PAP and D-H stains were cross checked with HE stain and there result showed similarity in staining Keratin on tissue sections of FFPE tissue blocks. For the other four parameters Mod PAP showed an excellent result but not D-H. The possible cause could be the interference of acetic acid with other DH staining solutions[32,33,34,35,36].

7. Strength and Limitation of the Study

7.1. Strength of the study

- ✓ In this study three pathologists were involved but related studies often used one evaluator.
- ✓ Senior Pathologist were used as a tie breaker
- ✓ Slides were evaluated with similar microscope i.e. Olympus.
- ✓ Larger sample size were used.
- ✓ Six pilot studies were carried out in this research. The last pilot study was done after research data’s were collected. Pilot studies enabled us to check the three stains in different types of tissue and cases. It gives a chance to improve staining by changing minor things without changing staining protocols.

7.2. Limitation of the study

- ✓ Lack of Published paper on Mod PAP and D-H stains on paraffin block tissue section.
- ✓ The gap observed on basic staining characteristics parameter of D-H stain were not checked using different kinds of nuclear or cytoplasmic stains.
- ✓ The number of Keratin special stains included were few.
- ✓ IHC procedures were not performed due to financial constraints.

8. Conclusion and Recommendation

8.1. Conclusion

In the present study, Mod PAP and D-H stains were cross checked with HE stain and there result showed similarity in staining Keratin on tissue sections of FFPE tissue blocks. For the other basic staining characteristics Mod PAP showed an excellent result. However, D-H showed inferior result perhaps it could be due to the interference of acetic acid with other DH staining solutions.

8.2. Recommendation

Mod PAP and D-H stains could support diagnosis if they are ordered on cases that need differentiation of mucin, pre-keratin and keratin.

Using this research as a reference, I recommend further study on D-H stain by modifying the method i.e. using different types of nuclear and cytoplasmic stains. It is also important to extend this research by using different types of Mod PAP stain or by adding other special stain. Further more, it would strengthen the new study if we add other tissue types like punch, lymph node and breast biopsies.

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APPENDIX A: Tissue processing schedule

(VIP-150 Tissue Tek II tissue processor schedule)

1. 4% Formalin-1 hour-35^{0}c
2. Water-5 second-35^{0}c
3. 50% Ethanol alcohol-1 hour-35^{0}c
4. 70% Ethanol alcohol-1 hour-35^{0}c
5. 80% Ethanol alcohol-1 hour-35^{0}c
6. 90% Ethanol alcohol-1 hour-35^{0}c
7. 100% Ethanol alcohol-1 hour-35^{0}c
8. 100% Ethanol alcohol-1 hour-35^{0}c
9. Xylene-1 hour-35^{0}c
10. Xylene-1 hour-35^{0}c
11. Paraffin wax -45 minutes-68^{0}c
12. Paraffin wax-45 minutes-68^{0}c
13. Paraffin wax-45 minutes-68^{0}c
14. Paraffin wax-45 minutes-68^{0}c

APPENDIX B: Solution Preparation Procedures

A. HE stain

1. Harri's hematoxylin

Hematoxylin…………2.5gm
Absolute alcohol……25ml
Potassium alum………50gm
Distilled water………..500ml
Sodium iodate…………0.5gm
Glacial acetic acid……...10ml

1. 0.3% Eosin Y solution

Eosin Y………………3gm
Distilled water……….1000ml

2. 0.3% Acid alcohol

HCL………………3ml
70% Acohol……….997ml

3. 0.5% Lithium carbonate

Lithium carbonate……….5gm
Distilled water……………1000ml

B. D-H stain

1. Mayer's hematoxylin

Hematoxylin……….1gm
Distilled water………1000ml
Potassium alum………50gm
Sodium iodate………..0.2gm
Citric acid……………..1gm
Chloral hydrate AR…..30gm

2. 1% Phloxine B

Phloxine B……………..10gm
Distilled water………….1000ml

3. 0.5% Alcian Blue

Alcian blue………….5gm
1.5% Acetic acid ……..1000ml

4. **Orange G-6**

    0.5% Solution in 95% alcohol……..1000ml
    Phosphotungistic acid……………….0.15gm

C. Mod PAP stain

1. **Harri's hematoxylin(similar to HE)**
2. **1% Phloxin B(similar to D-H)**
3. **Orange G-6(similar to D-H)**
4. **Eosin-Azur 50**

    Light green SF 0.5% solution in 95% alcohol……………450ml
    Bismarck brown 0.5% solution in 95% alcohol……….450ml
    Eosin Y 0.5% solution in 95% alcohol…………………100ml
    Phosphotungistic acid……………………………..….0.2gm
    Lithium carbonate, saturated solution ………………….10 drops
5. **0.3% Acid alcohol(similar to HE)**
6. **0.5% Lithium carbonate(similar to HE)**

APPENDIX C: Protocols of the three stains used in the Study

I. Hematoxylin and Eosin Stain

Staining procedure

1. Deparaffinize sections through 2 changes of xylene, absolute alcohol, and 95% alcohol to water wash.
2. Wash in distilled water for 10 min
3. Stain with Harris's hematoxylin stain for 7 min.
4. Wash slides in tap water and differentiate in 1% acid alcohol
5. Wash in tap water for 10 min and dip slides in lithium carbonate for bluing for 5 min
6. Stain slides with eosin for 15 sec.
7. Slides will be dehydrated with graded alcohol, cleared in xylene and mounted.

Results

Nuclei…………………………………………………Blue-Violet

Cytoplasm…………………………………………….. Blue-Violet

Keratin…………………………………………………Orange-red

Collagen……………………………………………….. Orange-red

Smooth muscle…………………………………………. Orange-red

Reference

- Ramulu S, Kale AD, Hallikerimath S, Kotrashetti V. Comparing modified papanicolaou stain with ayoub-shklar and haematoxylin-eosin stain for demonstration of keratin in paraffin embedded tissue sections. J Oral Maxillofac Pathol 2013;17(1):23-30.

II. Dane-Herman stain

Fixation: **10%** NBF

Sections: 6 microns

Solutions used:

- -Mayer’s hematoxylin
- -Orange G6
- -Alcian Blue
- Phloxine-B

Staining:

1. Deparaffinize sections through 2 changes of xylene, absolute alcohol, and 95% alcohol to water wash.
2. Mayer's Hematoxylin for 10 minutes. Blue in running tap water for 10 minutes. Rinse in distilled water.
3. Phloxine B Solution, 1% for 3 minutes. Wash in running tap water to remove excess stain. Rinse in distilled water.
4. Alcian Blue solution for 5 minutes. Wash in tap water for 2 minutes. Rinse in distilled water.
5. Orange G Solution for 13 minutes.
6. Transfer slides to 95% alcohol, two changes, five dips each; absolute alcohol, two changes, fifteen dips each.
7. Clear in xyelen & Mount.

Results

Acid Mucopolysaccharides ……………………………Blue

Prekeratin and Keratin …………………………………Orange to Red

Nuclei……………………………………………………Blue violet

Cytoplasm ……………………………………………Eosinophilic

Collagen………………………………………………Orange to light pink

Smooth muscle ………………………………………light pink

Reference

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III. *Modified papanicolaou stain*

Solutions used:

- -Harris's hematoxylin
- -Orange G6
- -Eosin – azure -50
- Phloxine-B

Procedure for staining

1. Deparaffinize sections through 2 changes of xylene, absolute alcohol, and 95% alcohol to water wash.
2. Stain in Harris's hematoxylin for 6 min
3. Rinse in two changes of tap water and dip in acid alcohol
4. Rinse thoroughly in tap water and dip in lithium carbonate
5. Wash in running tap water for 10 min, then rinse in distilled water
6. Stain in phloxine –B for 5 min
7. Rinse in distilled water and dehydrate
8. Stain in Orange G6 for 5 min
9. Rinse in 95% alcohol
10. Stain in eosin azure for 4 min
11. Rinse in 95% alcohol complete dehydration in absolute alcohol
12. Clear in xylene and mount.

Results

- Nuclei ……………………………………………………….Blue
- Acidophilic cells ……………………………………………Red
- Basophilic cells …………………………………………… Blue Green
- Erythrocytes ………………………………………………..Orange-red
- Keratin …………………………………………………….. Orange-red
- Superficial cells ……………………………………………. Pink
- Intermediate and Parabasal Cells …………………………… Blue Green
- Eosinophil …………………………………………………… Orange Red

Reference

- Ramulu S, Kale AD, Hallikerimath S, Kotrashetti V. Comparing modified papanicolaou stain with ayoub-shklar and haematoxylin-eosin stain for demonstration of keratin in paraffin embedded tissue sections. J Oral Maxillofac Pathol 2013;17(1):23-30.

APPENDIX D:

1. Staining score Checklist

Nuclear stain (Inadequate=0 and Adequate=1)

Cytoplasmic stain (Inadequate=0 and Adequate=1)

Keratin Pattern (Negative=0 and Positive=1)

Background stain (Absent=0 and Present=1)

Uniformity (Absent =0 and Present=1**)**

Operational Definition

Keratin Pattern: the presence of different keratin formation and maturation stages and how they respond to keratin special stains. This includes how keratin pearls and individual cell keratin respond to the stain.

- Keratin pattern is Negative=0 if keratin structure of tissue section is not stained orange-red.
- Keratin pattern is Positive=1 if keratin structure of tissue section is orange-red.

Nuclear detail: ability to show or stain different parts of nucleus and their division clearly.

- Nuclear detail is Inadequate=0 if the nuclear stain is granular, disintegrated and out of focus.
- Nuclear detail is Adequate=1 if the nuclear stain is round with clear membrane.

Cytoplasm transparency: ability to show or stain cytoplasm clearly and separately.

- Cytoplasmic transparency is Inadequate=0 if the cytoplasmic stain is disintegrated cytoplasmic, granular cytoplasm and out of focus.
- Cytoplasmic transparency is Adequate=1 if the cytoplasmic stain is intact cytoplasmic membrane and transparent membrane.

Background stain: the obscuring of any tissue part by stain other than the tissue nature. E.g. if the nucleus is stained by eosin or if cytoplasm is stained by hematoxylin.

- Background stain is Not present=0 if there is no background stain on nucleus, cytoplasm or keratin part of tissue section.
- Background stain is Present=1if there is background stain or unusual stain on nucleus, cytoplasm or keratin part of tissue section.

Uniformity of stain: sameness of stain throughout individual cell or tissue section.

- Uniformity of stain is Inadequate=0 if the tissue section is stained in different shades of colour in an individual cell.
- Uniformity of stain is Adequate=1 if the tissue section is uniformly stained throughout the individual cell

2. Stained tissue sections scoring table

S.No.	Criteria's	Slide A	Slide B	Slide C	Tissue Type	Remark
	Nuclear stain					
	Cytoplasmic stain					
	Uniformity					
	Background stain					
	Keratin Pattern					
	If Keratin pattern is positive; Is the maturation stage differentiable? If yes, please mention the name of the stage with the specific color the keratin gave.	Yes/No? Stage? ________ Color? ________	Yes/No? Stage? ________ Color? ________	Yes/No? Stage? ________ Color? ________		
	Is there keratin pearls? What is the color of the keratin pearl?	Yes/No? Stage? ________ Color? _______	Yes/No? Stage? ________ Color? _______	Yes/No? Stage? ________ Color? _______		

3. General question for Evaluators

1. What kind of contribution does the other two new stains gave?
2. Any comment (general opinion) about the difference and sameness of the three stain?
3. Any significant advantage the different stains add for tissue type or diagnosis in general?