Original Research Article

Comparison of Ayoub Shklar Stain, Dane Herman, Modified Pap and Routine Hematoxylin and Eosin Stain for the Keratin Identification

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Abstract

Introduction: Keratin constitutes the most abundant constituent of cytoskeleton of all epithelia and thus provides a mechanical support for the cells and nucleus. It forms the superficial most layer (stratum corneum) in keratinized epithelium of the oral cavity. Keratin may also be present in various pathologic conditions such as squamous cell carcinoma, verrucous carcinoma and odontogenic keratocyst.

Aim and objective: To determine and compare the staining pattern and intensity of special stains i.e. modified Papanicolaou, Dane-Herman method and Ayoub-Shklar with respect to routinely used Hematoxylin and eosin (H&E) stain.

Materials and methods: A retrospective study was carried out on 30 formalin-fixed, paraffin-embedded tissue blocks comprising of 10 cases of odontogenic keratocyst (OKC), 10 cases of verruca vulgaris (VV) and 10 cases of oral squamous cell carcinoma (OSCC) histopathologically diagnosed (using H&E) and retrieved from archives of the Department of Oral Pathology IDST College, Modinagar. All the sections were stained with Hematoxylin and eosin (H&E), modified Papanicolaou, Dane-Herman and Ayoub-Shklar stain.

Results: In the present study, staining pattern and intensity was better in special stains like modified Papanicolaou, Dane-Herman and Ayoub-Shklar as compared to Hematoxylin-eosin stain.
Conclusion: Keratin was well demonstrated by all the special stains such as modified Papanicolaou, Dane-Herman and Ayoub-Shklar as compared to Hematoxylin-eosin and can be used as an adjunct to routine staining methods in demonstrating keratin.

Key words

Staining pattern, Intensity, Keratin, Dane-Herman.

Introduction

Keratins are the most diverse group of proteins that belong to the family of intermediate filament (IF) and comprises about 80% of the total protein content in differentiated cells of stratified epithelia [1-3]. Keratins are defined as intermediate filament forming proteins, (10 nm in diameter) with specific physicochemical properties, produced in any vertebrate epithelia.
Keratins are the most abundant cellular proteins and constitute the principal component of the cytoskeleton of all epithelia as these intermediate filaments provide mechanical support for the cells and nucleus. It forms the superficial layer (stratum corneum) in keratinized epithelium in the oral cavity. Keratin may also be present in various pathologic conditions such as squamous cell carcinoma, verrucous carcinoma, odontogenic keratocyst etc. [4-5]. They play an important role in epithelial cell protection from mechanical and non-mechanical stressors. Keratins have a number of different benefits for use as marker proteins to distinguish epithelial tumors from mesenchymal tumors, both histologically as well immunohistochemically. In establishing a definitive diagnosis, it is seldom valuable to determine histologically the degree of keratinization or the presence and / or absence of keratin through the application of a stain which discerns keratin [6].

Hematoxylin and eosin (H&E) has always been considered as a gold standard in staining structures like collagen, amyloid, muscle, keratin, extracellular and intracellular secretions stain eosinophilic however, this staining technique has its drawbacks occasionally; for example, the color contrast cannot be assessed at all times, hence leads to uncertainty in diagnosis, especially in cases such as moderately or poorly differentiated squamous cell carcinomas, whereas it is also challenging to recognize the epithelial infiltration into the connective tissue and the keratin pearl formation [7].

Keratin can be determined with the help of Schiff's reagent along with various stains like Kreyberg's method, modified Papanicolaou, Dane-Herman and Ayoub-Shklar methods. Ayoub-Shklar stain is a quick and reliable histological marker used to determine the presence/absence and degree of keratinization in the paraffin embedded tissue sections. In this stain, keratin appears distinct red in color [8].

Papanicolaou stain is a polychromatic staining technique which stains preferentially on the basis of degree of cell maturity and cellular metabolic activity. The main objectives of this stain are good nuclear detail, differential counterstaining and cytoplasmic transparency [9]. The main use of orange G6 in the Papanicolaou stain is to stain keratin. Superficial cells with high content of keratin stain yellow-orange hue and parabasal cells stain green to blue in color [10]. Modified papanicolaou stain (PAP) was obtained by adding phloxine-B that is a red acid dye and stains prekeratin as well keratin distinctly red in color on paraffin embedded tissue sections. Hence, modified PAP stain can be used specifically to stain keratin [6]. Dane-Herman is used to stain prekeratin and keratin and appear as orange to red in colour [11]. The aim of the present study was to determine and compare the staining pattern and intensity of special stains i.e. modified Papanicolaou, Dane-Herman method

and Ayoub-Shklar with respect to routinely used Hematoxylin and eosin (H&E) stain.

Materials and methods

A retrospective study was carried out on 30 formalin-fixed paraffin- embedded tissue blocks comprising of 10 cases of odontogenic keratocyst (OKC), 10 cases of verruca vulgaris (VV) and 10 cases of oral squamous cell carcinoma (OSCC) histopathologically diagnosed (using H&E) and retrieved from archives of the Department of Oral Pathology IDST College, Modinagar, Uttar Pradesh, India in the year 2016-17. The study was approved by the Institutional Ethical Committee of IDST College, Modinagar.

The tissue samples were divided into three groups:-
Group 1 - OKC (10 cases)
Group 2 - VV (10 cases)
Group 3 - OSCC (10 cases)
From each paraffin embedded tissue block, four sections of 3-4 μm thickness were cut by using semiautomatic rotary microtome (Yorko) and the sections were lifted onto the glass slides for staining with Hematoxylin and eosin (H&E), modified Papanicolaou, Dane-Herman and Ayoub-Shklar stain.

Staining procedure:

A. Hematoxylin and eosin stain

1. The deparaffinized sections in xylene were dehydrated in various grades of alcohol for 5 minutes each.
2. After water wash for 10 min, the slides were stained with Harris's hematoxylin stain for 7 min.
3. Later, water washed for 10 min and after differentiation in acid alcohol, the slides were dipped in lithium carbonate for bluing for 5 min and were stained with eosin for 15 sec.
4. Dehydrated with graded alcohol, cleared in xylene and mounted.

B. Modified Papanicolaou stain

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

C. Ayoub-Shklar stain:

1. The sections were deparaffinized in xylene I and II for 5 min each. Later dehydrated in various grades of alcohol i.e. 90% and 70% and water washed for 10 min
2. Acid fuschin solution was added for 3 min
3. Aniline blue OG was added directly to the sections for 30 min
4. Sections were transferred to 95% alcohol – 2 changes
5. Sections were dehydrated, cleared and mounted.

D. Dane-Herman stain:

1. The deparaffinized sections in xylene were dehydrated in various grades of alcohol for 5 minutes each.
2. After water wash for 10 min, the slides were stained with Mayer's hematoxylin stain for 10 min. Blue in running tap water for 10 minutes. Rinse in distilled water.
3. Phloxine B solution (1 % aqueous) for 3 mins. Wash in running tap water to remove excess stain. Rinse in distilled water.
4. Alcian blue for 5 mins. Wash in tap water 2mins. 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 xylene and then mounting

After staining and mounting, all the stained slides were examined by two observers under the microscope to eliminate any subjective bias.

Evaluation of staining

All the stained sides were analyzed by a semi-quantitative scoring system for both staining pattern and intensity. The staining pattern was graded as:-

Score 0- No staining
Score 1- Patchy staining
Score 2- Uniform staining
Score 3- Patchy and uniform staining

The intensity of staining was graded as:-

Score 0- No staining
Score 1- Mild staining
Score 2- Moderate staining
Score 3- Intense staining

Statistical analysis

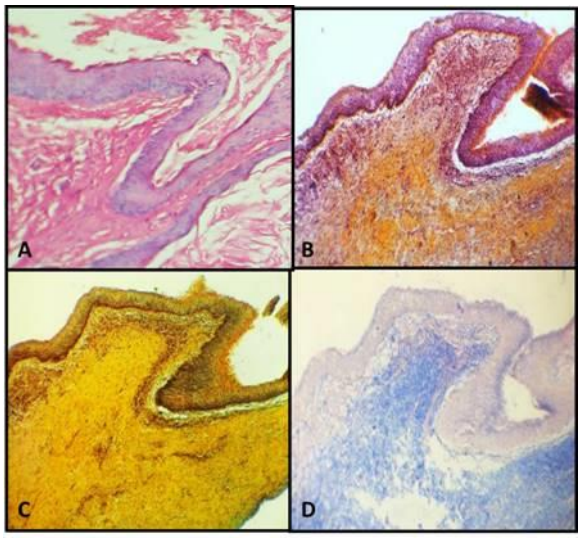
Data was obtained and statistically analyzed with the help of Statistical Package for Social Sciences (SPSS) software version 21.0 using percentage and Cronbach's alpha reliability test. A probability value of <0.05 was considered to be statistically significant.

Results

The photomicrographs of keratin stained with H and E and the special stains (i.e. Modified Papanicolaou, Dane-Herman, Ayoub-Shklar) in OKC, verruca vulgaris and OSCC were shown in **Figure - 1, 2, 3** respectively.

A good interobserver reliability was found on applying Cronbach's alpha reliability test to the observations obtained from all two observers for both staining pattern and intensity. The staining pattern of H & E and special stained sections in OKC, Verruca vulgaris and OSCC was shown in Table 1. In the study, 70%, 80% and 70% of the H & E stained sections of OKC, OSCC and verruca vulgaris revealed score 2. In modified Papanicolaou stained sections, 40%, 50% and 40% of the cases of OKC, verruca vulgaris and OSCC showed score 1, score 2 and score 1 respectively. In this study, 50%, 30% and 60% of the Dane-Herman stained sections of OKC, verruca vulgaris and OSCC revealed score 2, score 3 and score 2 respectively whereas 20%, 40% and 50% of the Ayoub Shklar stained sections of OKC, verruca vulgaris and OSCC showed score 3, score 1 and score 2 respectively. The p-value was found to be statistically significant (**Table - 1**).

Figure - 1: Photomicrograph of OKC (odontogenic keratocyst) in which keratin stained with H& E and special stains, A) Hematoxylin and eosin stained section (H & E), B) Modified Papanicolaou stained section, C) Dane-Herman stained section and D) Ayoub-Shklar stained section.



The staining intensity of H & E and special stained sections in OKC, Verruca vulgaris and OSCC was shown in **Table - 2**. The staining intensity was higher in modified Papanicolaou stained sections of OKC than that of Dane-Herman and Ayoub-Shklar stained sections. In verruca vulgaris cases, the staining intensity was higher in Dane-Herman stain followed by Ayoub-Shklar, modified Papanicolaou and Hematoxylin–eosin stain. In this study, the staining intensity was greater in Ayoub-Shklar in OSCC stained sections followed by Dane Herman and modified Papanicolaou. The p-value was found to be statistically significant (**Table - 2**).

Figure - 2: Photomicrograph of Verruca Vulgaris in which keratin stained with H& E and special stains, A) Hematoxylin and eosin stained section (H & E), B) Modified Papanicolaou stained section, C) Dane-Herman stained section and D) Ayoub-Shklar stained section.

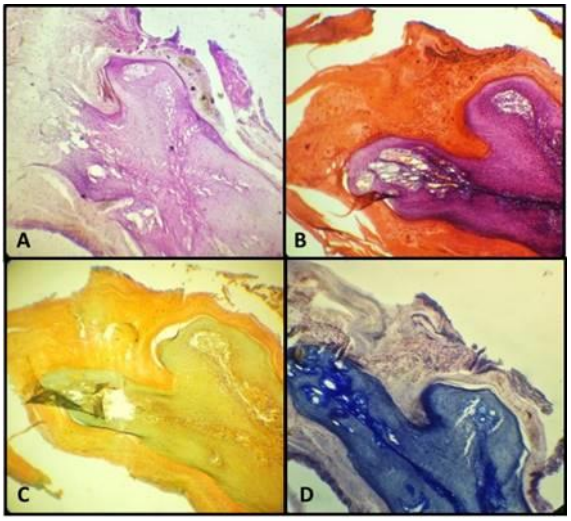


Figure - 3: Photomicrograph of Oral squamous cell carcinoma (OSCC) in which keratin stained with H& E and special stains, A) Hematoxylin and eosin stained section (H & E), B) Modified Papanicolaou stained section, C) Dane-Herman stained section and D) Ayoub-Shklar stained section.

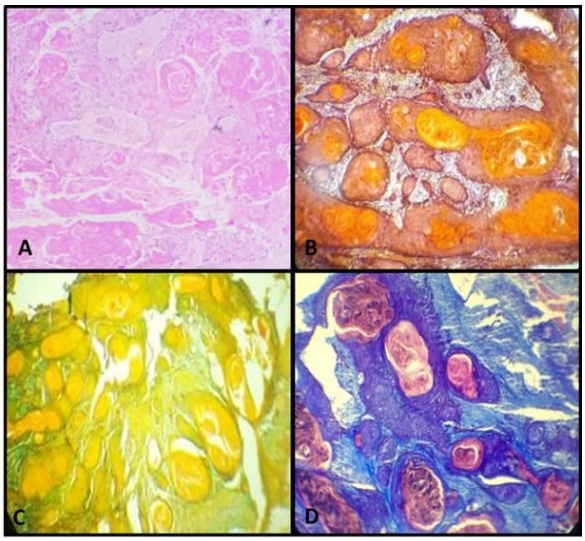


Table - 1: Staining pattern of Hematoxylin and eosin, Modified Papanicolaou, Dane-Herman and Ayoub-Shklar in Odontogenic keratocyst, verruca vulgaris and Oral squamous cell carcinoma.

Stain	**Staining pattern**				
	Odontogenic keratocyst				
	Score 0	**Score 1**	**Score 2**	**Score 3**	**P-value**
Hematoxylin and eosin	00 (0%)	03 (30%)	07 (70%)	00 (0%)	<0.001
Modified Papanicolaou	00 (0%)	04 (40%)	03 (30%)	03 (30%)	
Dane-Herman	00 (0%)	02 (20%)	05 (50%)	03 (30%)	
Ayoub-Shklar	00 (0%)	04 (40%)	04 (40%)	02 (20%)	
	Verruca vulgaris				
Hematoxylin and eosin	00 (0%)	02 (20%)	07 (70%)	01(10%)	0.001
Modified Papanicolaou	00 (0%)	02 (20%)	05 (50%)	03 (30%)	
Dane-Herman	00 (0%)	02 (20%)	05 (50%)	03 (30%)	
Ayoub-Shklar	00 (0%)	04 (40%)	03 (30%)	03 (30%)	
	Oral squamous cell carcinoma				
Hematoxylin and eosin	00 (0%)	02 (20%)	08 (80%)	00 (0%)	0.001
Modified Papanicolaou	00 (0%)	04 (40%)	03 (30%)	03 (30%)	
Dane-Herman	00 (0%)	02 (20%)	06 (60%)	02 (20%)	
Ayoub-Shklar	00 (0%)	03 (30%)	05 (50%)	02 (20%)	

Score 0- No staining, Score 1- Patchy staining, Score 2- Uniform staining and Score 3- Patchy and uniform staining

Table - 2: Staining intensity of Hematoxylin and eosin, Modified Papanicolaou, Dane-Herman and Ayoub-Shklar in Odontogenic keratocyst, verruca vulgaris and Oral squamous cell carcinoma.

Stain	Staining Intensity				
	Odontogenic keratocyst				
	Score 0	Score 1	Score 2	Score 3	P-value
Hematoxylin and eosin	00 (0%)	02 (20%)	08 (80%)	00 (0%)	0.001
Modified Papanicolaou	00 (0%)	04 (40%)	03 (30%)	03 (30%)	
Dane-Herman	00 (0%)	02 (20%)	06 (60%)	02 (20%)	
Ayoub-Shklar	00 (0%)	03 (30%)	05 (50%)	02 (20%)	
	Verruca vulgaris				
Hematoxylin and eosin	00 (0%)	02 (20%)	07 (70%)	01(10%)	0.001
Modified Papanicolaou	00 (0%)	02 (20%)	06(60%)	02 (20%)	
Dane-Herman	00 (0%)	01 (10%)	05 (50%)	04 (40%)	
Ayoub-Shklar	00 (0%)	03 (30%)	04 (40%)	03 (30%)	
	Oral squamous cell carcinoma				
Hematoxylin and eosin	00 (0%)	03 (30%)	07 (70%)	00 (0%)	0.001
Modified Papanicolaou	00 (0%)	04 (40%)	04 (40%)	02 (20%)	
Dane-Herman	00 (0%)	02 (20%)	05 (50%)	03 (30%)	
Ayoub-Shklar	00 (0%)	04 (40%)	02 (20%)	04 (40%)	

Score 0- No staining, Score 1- Mild staining, Score 2- Moderate staining, Score 3- Intense staining

Discussion

Keratin constitutes the most abundant constituent of cytoskeleton of all epithelia and thus provides a mechanical support for the cells and nucleus. Keratin plays a great role as a marker protein in determining a definitive histological diagnosis, to distinguish between the epithelial and mesenchymal tumors and in certain conditions, when the epithelial component may be scant and can be identified only by the presence of keratin reactivity [6, 12].

Special stains are the stains which are used to visualize specific tissues and cellular structures. These are the dyes that bind to the cellular components either physically or by chemical bonds. Various special histochemical stains that are used to stain keratin are Ayoub-Shklar, Dane Herman method, Schiff's reagent by oxidation with performic acid etc. These stains may highlight small foci of abnormal keratinisation that occasionally missed in routine H&E. These stains, highlights even the minute areas of keratin that can be missed by routine H and E staining. Keratins are also brilliantly stained red and orange to magenta shades respectively by phloxine, component of Dane-Herman method, and orange G dye, component of both Ayoub-Shklar and Papanicolaou stain [13]. The present study was done to determine and compare the staining pattern and intensity of special stains i.e. modified Papanicolaou, Dane-Herman method and Ayoub-Shklar with respect to routinely used Hematoxylin and eosin (H&E) stain.

In the present study, staining pattern and intensity was better in special stains like modified Papanicolaou, Dane-Herman and Ayoub-Shklar as compared to Hematoxylin-eosin stain.

A study carried out by Rao, et al. [14] have shown that Ayoub Shklar method was better than PAP, Dane Herman, Gram's and modified Alcian blue PAS method in terms of staining intensity and equal efficacy in demonstrating type of keratin with all the stains. Ramulu, et al. [8] have shown in their study that all stains were efficient in staining keratin but H and E stain was

better in demonstration of keratin pearls in oral squamous cell carcinoma cases. In OKC cases, the staining intensity was higher with modified PAP followed by Dane-Herman and Ayoub-Shklar as compared to H & E. In verruca vulgaris, the staining intensity was greater with Dane-Herman followed by Ayoub-Shklar and modified PAP than that of H & E. In OSCC cases, staining intensity was better with Ayoub-Shklar followed by Dane-Herman and modified PAP than that of H & E. Keratin was well demonstrated by all the special stains such as modified Papanicolaou, Dane-Herman, Ayoub-Shklar etc. and can be used as a useful adjunct to routine staining methods in determining keratin. The main limitation of our study was that the sample size was small and we would not differentiate the keratin type. Further studies on larger sample size should be carried out using keratin specific histochemical stains, and should also be correlated with the keratin type to appreciate the uneven staining intensity and pattern.

Conclusion

In the present study, keratin was well demonstrated by all the special stains such as modified Papanicolaou, Dane-Herman and Ayoub-Shklar as compared to Hematoxylin-eosin on the basis of staining pattern and intensity. Hence, all the special stains can be used as an adjunct to routine staining methods in demonstrating keratin.

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