Original Article

"Ayoub-Shklar" as a Special Stain for Keratin: A Histopathological Study

Raman Dineja[1], Harikrishnan Prasad[2], KenniyanKumar Sri Chinthu[2], Muthusamy Rajmohan[2]

[1]Department of Oral Pathology and Oral Microbiology, SRM Dental College, Chennai, [2]Department of Oral Pathology and Oral Microbiology, KSR Institute of Dental Science and Research, Tiruchengode, Tamil Nadu, India

Abstract

Background: Special stains in histopathological studies are used to identify the structures with different dyes apart from the routine stain hematoxylin and Eosin (H and E). The component which is present in the special stains will have a specific bond and affinity for particular tissue components in the histological specimen. Structures like keratin takes up an eosinophilic stain in routine (H and E) staining. Most of the potentially malignant disorders and carcinomas arise due to the keratinization defect, which makes keratin an important diagnostic tool. There are different stains such as Ayoub-Shklar (A-S), Dane-Herman (D-H), and rapid Papanicolaou (PAP) which is used to identify keratin. In A-S stain, keratins can be stained in magenta-red and orange colors. **Aim:** we compared A-S special stain and routine stain in terms of staining intensity or quality, the pattern of staining, and specificity for staining keratin. **Materials and Methods:** Thirty cases from the department archives that included 10 well-differentiated squamous cell carcinoma, 10 verrucous carcinoma, and 10 epithelial dysplasia were taken and each case was stained with both A-S and H and E stain. **Results:** A-S showed an almost equal distribution of uniform and patchy staining patterns, but H and E showed more patchy staining patterns in the three groups. H and E stain shows good staining quality than A-S. A-S shows almost 90% of satisfactory staining specificity. **Conclusion:** Special stain like A-S stain can be used to stain keratin in different color, but H and E always remain gold standard stain.

Keywords: Ayoub-Shklar stain, keratin, special stain

Introduction

Special stains help to differentiate a structure from the surrounding structures with more details that cannot be appreciated in routine hematoxylin and eosin (H and E) staining. In some cases, certain tissues will not take up the routine stain, so there comes the role of some special stains to identify them.[1] In oral epithelium, keratins are said to be the major protein, which is found in greater concentration and also with diversity in the keratinocytes as same as in the skin.[2] Keratin may also be involved in many pathological conditions such as epithelial malignancies, odontogenic keratocyst, and epithelial dysplasia conditions. Keratin is known to be a very useful protein marker to distinguish between epithelial tumors from mesenchymal tumors. Degree of keratinization and keratin pearl formation helps to differentiate the squamous cell carcinoma (SCC) as well differentiated or moderately or poorly differentiated. H and E staining is considered the gold standard staining method for keratin, amyloid, and collagen, but with some drawbacks like color assessments which make its identification difficult, sometimes it is also challenging in demonstrating infiltration of the epithelium into connective tissue and formation of keratin pearls.[3] Hence in that cases, it can be identified with the help of different stains such as Kreyberg's method of staining and Modified Papanicolaou (PAP). Whereas Ayoub-Shklar (A-S) stain is said to be a quick histological marker. This stain is used to indicate and evaluate the presence and degree of keratinization in the tissue sections. In this special stain, keratin will appear in red in color and in orange or in magenta color too.[4] In this

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Address for correspondence: Dr. Raman Dineja, Department of Oral Pathology and Oral Microbiology, SRM Dental College, Ramapuram, Chennai - 600 089, Tamil Nadu, India.
E-mail: dinejar@srmist.edu.in

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study, we decided to compare the efficiency of special stains for keratin and routine stain in demonstrating keratin.

Materials and Methods

This was a retrospective study and it was decided to carry out in 30 tissue blocks from the department archives. Ethical approval/consent is not required/waived off as per the Institutional ethical committee norms. Study samples comprising histopathologically diagnosed 10 cases of verrucous carcinoma, 10 cases of well-differentiated SCC (WDSCC), and 10 cases of epithelial dysplasia. The study samples were taken into three groups. Group 1 comprises 10 cases of WDSCC, Group 2 comprises 10 cases of VC, and Group 3 comprises 10 cases of epithelial dysplasia. Two sections from tissue blocks of 3 to 4 µm of tissue were sliced using a microtome. Each one of the sections was stained with routine stain and A-S stain.

Preparation and staining procedure of Ayoub-Shklar stain

Preparation involves solution A and solution B.

Solution A – contains acid fuchsin of 5 g and distilled water of 100 ml. Solution B – contains Orange G of 2 g, phosphotungstic acid of 1 g, and distilled water of 100 ml.

The staining procedure of A-S is followed as deparaffinized tissue sections were placed in xylene for 5 min and slides were dehydrated in different grades of alcohol and washed in tap water for 10 min. Slides were then placed in solution A for 3 min. Moreover, Solution B was added directly to the slides for 30 min. Later, slides were dehydrated, cleared, and mounted [Figure 1].

Hematoxylin and eosin staining procedure

The deparaffinization and dehydration is carried out for all the sections. Then rinsed with tap water for 10 min, the slides were stained with hematoxylin for 7 min then acid alcohol is used for differentiation; bluing was done for few minutes and stained with eosin stain for <10s. Cleared in xylene and mounted. Finally, slides were examined. Evaluation of staining was done by two observers with the following scoring criteria. For pattern of staining: uniform staining - 1, patchy staining - 2; for quality/intensity and specificity of staining, the scoring criteria where poor staining is scored as 0, satisfactory staining as 1, Good staining as 2, and score 3 for excellent staining.

Statistical analysis

Data were obtained, and a statistically method called Chi-square test was done with Statistical Package for the Social Sciences.

Results

When all samples were compared together, quality ($P = 0.004$), specificity ($P = 0.005$) was significantly better in H and E. In Group 2, Quality ($P = 0.05$) and specificity ($P = 0.02$) were significantly better in H and E stained sections. Keratin stained with H and E and A-S stain in WDSCC cases and in verrucous carcinoma and in epithelial dysplasia cases is shown in Figure 2a-f.

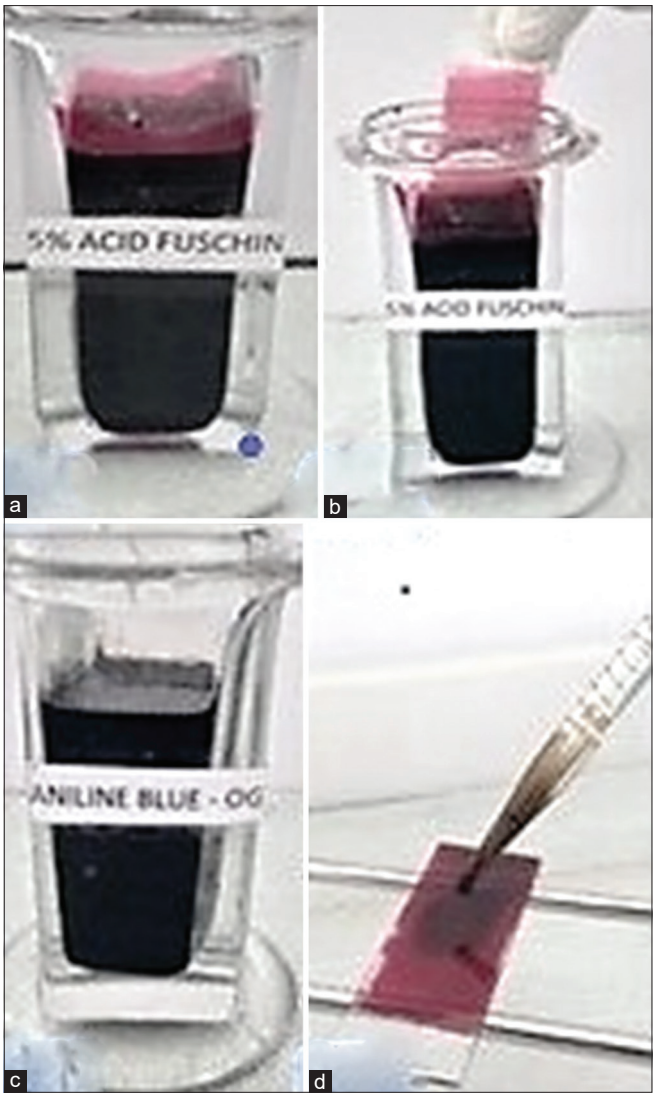

Figure 1: Staining procedure for Ayoub Shklar stain. (a) 5% Acid Fuschin, (b) slide placed in 5% Acid Fuschin, (c) Aniline Blue – OG stain, (d) Direct application of Aniline blue -OG stain over the slide

Comparison between groups was not made since three different lesions were taken with different amount of keratins.

Figure 3 shows the comparison of the staining pattern of A-S and H and E in all three groups, in that H and E stain showed 73% of patchy staining pattern and 26.6% of uniform staining, whereas A-S stain showed 43.3% of uniform staining pattern. A-S stains shows almost equal distribution of uniformity and patchy staining pattern. H and E show a more patchy staining pattern than A-S stain. Figure 4 shows the Quality or intensity of the staining pattern of H and E and A-S in all three groups. In this comparison, it indicates that 76.6% of H- and E-stained slides showed good quality of staining and 40% of A-S stained showed good staining quality. About 23% of H and E showed satisfactory staining quality, while 60% of A-S showed satisfactory staining quality. This indicates

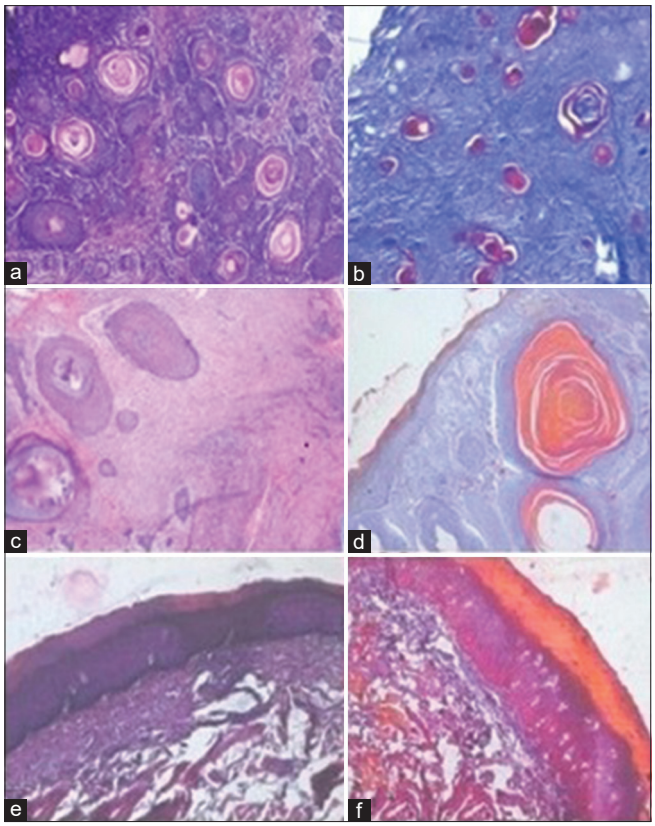

Figure 2: Histopathological images of hematoxylin and eosin (H and E) and Ayoub-Shklar (A-S) stain (×100). Photomicrograph of well differentiated squamous cell carcinoma in which keratin is stained with H and E (a) and A-S stain (b), Verrucous carcinoma in which keratin is stained with H and E (c) and A-S stain (d), Epithelial dysplasia in which keratin is stained with H and E (e) and A-S stain (f)

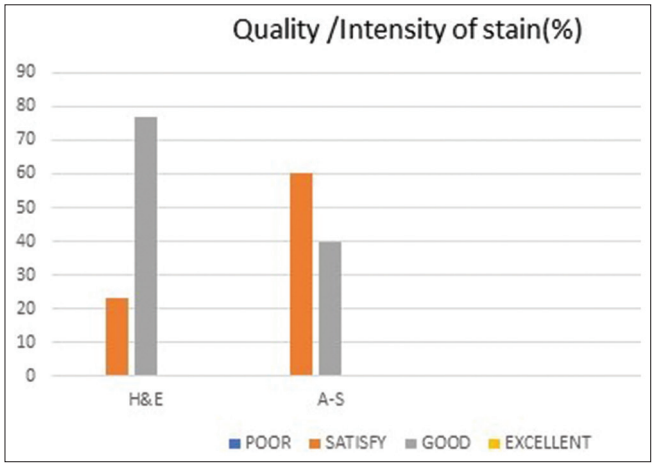

Figure 4: Graph representing the comparison of quality of staining in hematoxylin and eosin and Ayoub-Shklar. H and E: Hematoxylin and eosin, A-S: Ayoub-Shklar

that H and E stain shows good staining quality than A-S, but A-S stain also showed 40% of good staining and almost 60% of satisfactory staining quality. This difference in quality can be due to any pitfall in the staining procedure or during the preparation of stain or due to any artifacts. Figure 5 shows

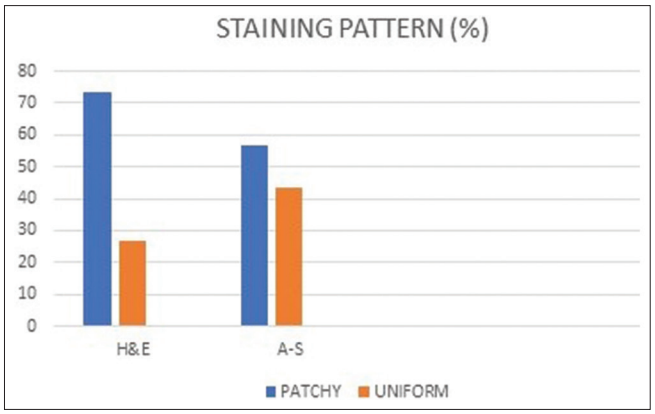

Figure 3: Graph representing the comparison of staining pattern in hematoxylin and eosin and Ayoub-Shklar. H and E: Hematoxylin and eosin, A-S: Ayoub-Shklar

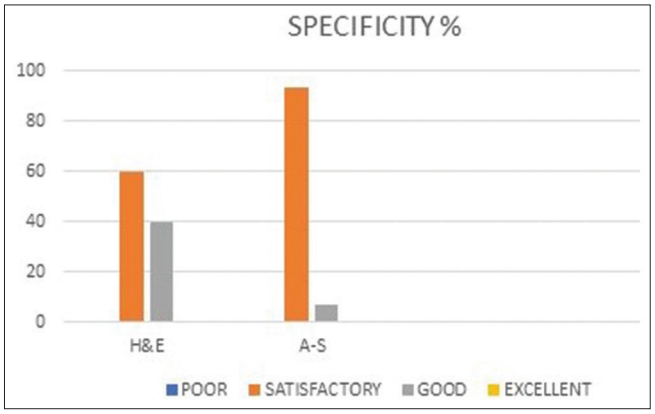

Figure 5: Graph representing the comparison of specificity in hematoxylin and eosin and Ayoub-Shklar. H and E: Hematoxylin and eosin, A-S: Ayoub-Shklar

the staining specificity of H and E and A-S in all three groups. Sixty percent of H and E shows satisfactory staining specificity and 40% showed good staining specificity, whereas A-S showed 90% of satisfactory staining specificity but only 6.7% of good staining specificity. Here, A-S shows almost 90% of satisfactory staining specificity, which indicates that A-S stains specifically keratin, but it may also stain some other structures; this may be due to the charges of the dye and structures to observe it.

Discussion

Oral epithelium acts as a primary barrier to the oral cavity. It is generally lined by epithelium, which is a simple stratified squamous type with tightly packed cells arranged in the form of layers. It helps to maintain structural integrity by continuous renewal of cells.[5] Because of the keratin formation, the surface layer in the epithelium of the hard palate, the gingiva is tough and abrasion resistant. For all epithelium, keratin forms a major component that helps in supporting the cells.[2]

When special and differential stains are applied to histological and cytological preparations, it will help to differentiate

specific cells, or tissue components, and in some cases, even microorganisms can be differentiated from the surrounding structures. These special stains are not routine stains. In the case of keratin, the special stain is recommended to identify and to know the importance of small foci of abnormal keratinization.[6] In conditions like oral submucous fibrosis or hyperkeratosis nonkeratinized epithelium will show keratinization.[7] Biopsy helps to detect the malignant changes in submucous fibrosis and also to consider its rate of transformation to malignancy.[8] Special stains for keratin can be used in oral submucous fibrosis cases to identify hyperkeratosis. Special stains are used to bind with the tissues and to some of the respective cellular components either by certain physical and chemical bonds. Special stains like A-S, Schiff's reagent are used to stain keratin specifically. These special stains will highlight even the small portion of epithelial differentiation that sometimes might be missed during routine H and E staining.[2] In this study, we compared the A-S with H and E to evaluate the staining quality or intensity, staining pattern and specificity of A-S to stain keratin in three different groups. In A-S stain, the keratin was stained as bright red, orange and also with different shades of magenta; this is mainly due to the component orange G dye. When comparing the staining pattern between the two stains, it shows that 73% of H and E stain showed patchy staining patterns, whereas 26.6% showed uniform staining. Whereas 43.3% in A-S stain showed uniform staining pattern. In a study done by Ramulu *et al*.,2013 found that all of their study samples showed uniform staining patterns in H and E, while in A-S stain only 8 cases showed patchy and 12 cases showed uniform staining pattern.[4] Corresponding to this, our study results also show less uniformity in the staining pattern of A-S. However, H and E showed uniform staining pattern of about 26% when compared to A-S it may be due to any procedural error or due to sectioning error. A Pilot study done by Zafar *et al*. to determine the staining specificity and intensity of A-S stain with routine H and E in KCOT cases. They found that special (A-S) stain showed better staining specificity and good staining quality. But because of less study sample, they did not conclude in terms of comparison with H and E.[2] In terms of quality or intensity, the present study shows that 76.6% of H- and E-stained slides showed good quality of staining and 40% of A-S stained showed good staining quality. About 23% of H and E showed satisfactory staining quality, while 60% of A-S showed satisfactory staining quality. This indicates that H and E stain shows good staining quality than A-S, but A-S stain also showed 40% of good staining and almost 60% of satisfactory staining quality. This difference in quality can be due to any pitfall in staining procedure or during the preparation of stain. In a study done by Rao *et al*., in 2015, they stained ortho and parakeratinized normal gingival tissue and found that the intensity of staining of A-S was higher than H and E.[6] The specificity of stains was also compared, which shows 60% of H and E showed satisfactory staining specificity and 40% showed good staining specificity, whereas A-S showed 90% of satisfactory staining specificity, but only 6.7% of good staining specificity. Here, A-S shows almost 90% of satisfactory staining specificity, which indicates that A-S stains specifically keratin, but it may also stain some other structures. A study done by Rao *et al*., in 2015, evaluated in terms of specificity, found H and E was better than special stain because not all the keratin in WDSCC undergo proper keratinization, which might be the reason when some poorly or nondifferentiated keratins pearls which makes them less reactive to special stain.[6]

In a study done by Anthwal *et al*. conducted a study to compare A-S stain, Dane-Herman (D-H), modified Pap and routine H and E stain for the keratin identification. In that study, they found that staining pattern and intensity was better in special stains such as modified PAP, D-H, and A-S when compared to H and E stain. Corresponding to our study A-S 43% of cases showed uniform staining pattern. And also, in our study only 40% of cases showed good staining quality or intensity which is not in accordance with their study,[3] Srivastava *et al*. conducted a comparative study to evaluate the special stain for keratin identification using modified Kreyberg's, modified PAP and A-S stain, they found these special stains can be used as an alternate stain for keratin. Differences in our result may be due to the small sample size compared to our study.[9] Kakkar *et al*. conducted a study to demonstrate keratin by using A-S, D-H, Alcian blue-PAS, Rapid Pap stain, and Gram's stain with H and E stain. They found that H and E showed better staining when compared to other special stain this result was similar. They also found that among different stains A-S stain showed better results.[10] Tharani and Wadhwani conducted a study to demonstrate ghost cells in calcifying odontogenic cyst; they used A-S, Van-Gieson, Mallory stain, H and E and found that A-S, and Mallory was good in differentiating ghost cells when compared to H and E and Van Gieson stain.[11] A-S stain was used to stain Toto bodies in inflammatory and reactive lesions, A-S was found to have high staining intensity and also showed a diffuse distribution that indicated Toto bodies originated from keratin.[12] Micro metastasis can also be detected using special stain in oral SCC.[13]

Conclusion

H and E continuous to be as gold standard for the identification of keratin in most of the circumstances. However, special stain like A-S stain can be used to stain them to distinguish keratin in different color. Yet many studies with more sample sizes and different cases need to be done to overcome the pitfalls.

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Conflicts of interest

There are no conflicts of interest.

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