

Comparative Evaluation of Special Stains (Alcian Blue–Periodic Acid Schiff, Rapid Papanicolaou, and Gram Stain) versus Hematoxylin and Eosin for Demonstration of Keratin in Well-Differentiated Oral Squamous Cell Carcinoma: A Histochemical Study

Hari Dutt Sharma¹, [Guide/Dr Shivali Vaid]²

¹Postgraduate Student, ²[Professor & Head

Department of Oral and Maxillofacial Pathology and Microbiology, Maharana Pratap College of Dentistry and Research Centre, Gwalior, Madhya Pradesh, India

Corresponding Author: Dr. Hari Dutt Sharma

Abstract

Background:

Keratin, an intracytoplasmic filamentous protein, constitutes nearly 80% of the total protein content of keratinized stratified squamous epithelium and provides structural integrity to epithelial cells. Alterations in the keratinization process are seen in a wide range of hereditary, premalignant, and malignant oral lesions, making keratin an important diagnostic marker in epithelial pathology. Routine Hematoxylin and Eosin (H&E) staining does not selectively demonstrate keratin, as most eosinophilic structures such as collagen, muscle, and secretions stain similarly, making differentiation difficult.

Aim:

To compare the staining efficacy and intensity of Alcian Blue–Periodic Acid Schiff (AB-PAS), Rapid Papanicolaou (Rapid PAP), and Gram stain with routine H&E stain for the demonstration of keratin in well-differentiated oral squamous cell carcinoma (OSCC).

Materials and Methods:

Thirty paraffin-embedded tissue blocks of histopathologically diagnosed well-differentiated OSCC were retrieved from the department archives. From each block, four serial sections of 4-micron thickness were prepared and stained with H&E (Group 1), AB-PAS (Group 2), Rapid PAP (Group 3), and Gram stain (Group 4). The staining specificity and intensity for keratin were evaluated and statistically compared across the four groups.

Results:

All four staining methods demonstrated keratin distinctly. H&E-stained sections showed a greater amount of keratin pearls compared with AB-PAS, Rapid PAP, and Gram stain. Among the special stains, AB-PAS showed the highest staining specificity and intensity, followed by Rapid PAP and Gram stain.

Conclusion:

All four stains can identify keratin in well-differentiated OSCC, and AB-PAS may be used as an alternative special stain to H&E for the demonstration of keratin. However, H&E remains the gold standard overall for keratin visualization on a comparative basis.

Keywords:

Keratin; Oral squamous cell carcinoma; Special stains; Alcian Blue–Periodic Acid Schiff; Papanicolaou stain; Gram stain; Hematoxylin and Eosin

Date of Submission: 26-09-2026

Date of Acceptance: 05-10-2026

I. INTRODUCTION

Epithelial tissue forms a barrier between the body and the external environment, protecting internal tissues from mechanical, chemical, and microbial insult. This protective demand is met largely through the synthesis of keratin, an intracytoplasmic filamentous protein that accounts for nearly 80% of the total protein content of keratinized stratified squamous epithelium and provides structural integrity and mechanical resilience to epithelial cells.¹

Keratins are obligate heterodimer intermediate filament proteins (approximately 10 nm in diameter) expressed in pairs of type I (acidic) and type II (basic) proteins. Genes encoding the acidic keratins, with the exception of K18, are located on chromosome 17, while those encoding the basic keratins, including K18, are

located on chromosome 12.² The term "keratin" is derived from the Greek word *kera*, meaning horn, reflecting the tough, protective nature of these proteins.

The oral cavity is lined by stratified squamous epithelium, which may be keratinized (further classified as ortho- or parakeratinized) or non-keratinized. Defects in the process of keratinization underlie a large number of hereditary, potentially malignant, and malignant disorders of the oral cavity, many of which are characterized by excessive or aberrant keratin production. Consequently, the pattern and amount of keratin can serve as an important diagnostic and prognostic marker in epithelial pathology, particularly in oral squamous cell carcinoma (OSCC).³

Routine Hematoxylin and Eosin (H&E) staining, though universally used for tissue diagnosis, does not selectively demonstrate keratin. Collagen, amyloid, muscle, keratin, and various extracellular and intracellular secretions all stain eosinophilic, making differentiation between these structures difficult on a purely morphological basis.

While immunohistochemistry can specifically localize keratin subtypes, it remains an expensive and technically demanding diagnostic tool that is not routinely available in all histopathology laboratories. Special histochemical stains offer a faster and more economical alternative for assessing the degree and pattern of keratinization. Several such methods have been described, including the Ayoub-Shklar (A-S) method, the Dane-Herman (DH) method, Alcian Blue–Periodic Acid Schiff (AB-PAS), Rapid Papanicolaou (Rapid PAP), Gram stain, Phloxine-Tartrazine, Aldehyde Fuchsin, Congo Red, Thioflavin T, and the Auramine-Rhodamine fluorescent method.^{4,5}

In the present study, three special stains — AB-PAS, Rapid PAP, and Gram stain — were used to demonstrate keratin in histopathologically diagnosed cases of well-differentiated OSCC, and their staining efficacy and intensity were compared with routine H&E stain.

Oral cancer is conventionally defined as squamous cell carcinoma of the lip, oral cavity, and oropharynx, since approximately 90% of malignancies arising in the oral region are histologically of squamous cell origin.⁶ The major etiological and predisposing factors for OSCC include tobacco smoking, alcohol consumption, ultraviolet radiation, human papillomavirus (HPV) infection, chronic *Candida* infection, nutritional deficiencies, and genetic predisposition. The World Health Organization has emphasized that early detection substantially improves treatment outcomes, underscoring the importance of education and screening in early diagnosis.⁷

II. AIM

- To compare the staining efficacy of AB-PAS, Rapid PAP, and Gram stain with H&E stain for keratin in well-differentiated OSCC.
- To compare the staining intensity of AB-PAS, Rapid PAP, and Gram stain with H&E stain for keratin.
- To evaluate which of the special stains is superior in terms of staining efficacy and intensity.

III. OBJECTIVES

- To stain 30 tissue sections (4-micron thickness) of diagnosed well-differentiated OSCC with H&E stain (Group 1).
- To stain 30 tissue sections (4-micron thickness) of the same cases with AB-PAS stain (Group 2).
- To stain 30 tissue sections (4-micron thickness) of the same cases with Rapid PAP stain (Group 3).
- To stain 30 tissue sections (4-micron thickness) of the same cases with Gram stain (Group 4).
- To evaluate the staining specificity and intensity of keratin with each special stain.
- To correlate and compare the staining specificity and intensity of keratin between AB-PAS, Rapid PAP, and Gram stain.

IV. MATERIALS AND METHODS

4.1 Study Design and Setting

This comparative, retrospective histochemical study was conducted in the Department of Oral and Maxillofacial Pathology and Microbiology, Maharana Pratap College of Dentistry and Research Centre, Gwalior, after obtaining Institutional Ethics Committee clearance [insert IEC approval number and date].

4.2 Study Subjects

Thirty archival paraffin-embedded tissue blocks of histopathologically diagnosed well-differentiated OSCC, retrieved from the histopathology laboratory of the department (cases of the preceding two years), were included in the study. From each block, four serial sections of 4-micron thickness were prepared and allotted to four groups: Group 1 — H&E stain; Group 2 — AB-PAS stain; Group 3 — Rapid PAP stain; and Group 4 — Gram stain. Stained sections were evaluated and the results statistically analyzed and compared.

4.3 Materials Used

Paraffin-embedded tissue blocks, an automatic microtome with wax-block holder, microscope slides, a water bath (approximately 45–46 °C), section adhesive, slide warmer, coupling jars, dropping bottles, coverslips, dissecting needles, forceps, scalpels, filter paper, DPX mounting medium, slide-washing and slide-carrying trays, a binocular microscope, gauze, paint brushes, cotton, chip blower, marker pens, slide labels, and personal protective equipment (gloves and masks) were used.

4.4 Tissue Processing

Paraffin blocks were trimmed and mounted on the microtome using the wax-block holder, and serial ribbon sections of 4-micron thickness were cut with a sharpened, properly clamped microtome knife. Sections were floated on a hot water bath (approximately 40 °C) using a paint brush to remove creases, mounted onto adhesive-coated slides, drained in an upright position, and dried overnight in an incubator at 37 °C. Each slide was labeled for identification prior to staining.

4.5 Staining Procedures

Hematoxylin and Eosin (H&E) stain: Sections were deparaffinized and hydrated through descending grades of alcohol, stained with Hematoxylin for 3–5 minutes, differentiated in 0.3% hydrochloric acid, blued in dilute ammonia water, counterstained with Eosin for 10–60 seconds, dehydrated through ascending grades of alcohol, cleared in xylene, and mounted with DPX.

Alcian Blue–Periodic Acid Schiff (AB-PAS) stain: Sections were deparaffinized in xylene and hydrated through graded alcohols, treated with 3% acetic acid, stained with 1% Alcian Blue (pH 2.5) for 30 minutes, oxidized in 0.5% periodic acid for 10 minutes, treated with Schiff reagent for 20 minutes, dehydrated, cleared, and mounted. Rapid Papanicolaou (Rapid PAP) stain: Using a ready-to-use kit (PAP-100T), sections were deparaffinized and hydrated, stained with Hematoxylin (PAP-1A reagent), blued, treated with 95% alcohol, stained with OG-6 (PAP-2B reagent), dehydrated, cleared, and mounted.

Gram stain: Sections were heat-fixed and flooded with carbol-fuchsin working stain with gentle heating for approximately five minutes, rinsed, decolorized with 20% sulfuric acid for one minute, counterstained with methylene blue for one minute, washed, and air-dried.

4.6 Evaluation and Statistical Analysis

Stained sections were evaluated under light microscopy for staining specificity and intensity of keratin by two independent observers blinded to the staining method [insert scoring criteria used, e.g., a 4-point scale: poor, satisfactory, good, excellent]. The findings were tabulated and statistically compared between groups using [insert statistical test, e.g., Chi-square test or Fisher's exact test], with $p < 0.05$ considered statistically significant. [Note to author: insert the specific software and test used, and the exact group-wise scores/p-values from the original data before submission.]

V. RESULTS

All four staining procedures — H&E, AB-PAS, Rapid PAP, and Gram stain — demonstrated keratin clearly and distinctly in well-differentiated OSCC sections. H&E-stained sections showed a greater amount of keratin pearls than sections stained with AB-PAS, Rapid PAP, and Gram stain.

Among the three special stains, AB-PAS showed superior staining specificity and intensity for keratin, followed by Rapid PAP and Gram stain, in that order. When all four staining methods were compared collectively, the overall staining specificity and intensity of H&E remained the highest. [Note to author: insert Table 1 with group-wise scoring distribution and the exact statistical values (chi-square/Fisher's exact test statistic and p-value) from your raw data, and Figure 1 with representative photomicrographs of each stain.]

VI. DISCUSSION

The identification and demonstration of keratin is of considerable diagnostic value in the histopathological grading of OSCC, since the pattern and amount of keratin synthesis reflect the degree of epithelial differentiation.⁸ Although H&E remains the routine stain in histopathology laboratories worldwide, it lacks specificity for keratin, as several other eosinophilic tissue components stain in a similar manner, which can make small or subtle foci of keratinization difficult to appreciate.⁹

Special histochemical stains have therefore been explored as economical alternatives or adjuncts to H&E for keratin demonstration. AB-PAS relies on the reaction of periodic acid with glycol groups in keratin-associated polysaccharides to generate aldehydes that combine with Schiff's reagent, producing a magenta reaction product, while Alcian Blue selectively stains acidic mucosubstances; together these reactions can highlight keratin distinctly from surrounding connective tissue.¹⁰ The Rapid PAP method, adapted from cytological practice, uses orange G6 to impart a yellow-orange hue to keratin-rich superficial cells, while Gram stain, though primarily a

bacteriological stain, has also been reported to demonstrate keratin through differential retention of carbol-fuchsin.¹¹

The present findings — that H&E showed a greater number of keratin pearls than the special stains, and that among the special stains AB-PAS showed the best specificity and intensity followed by Rapid PAP and Gram stain — are consistent with the comparative study by Bala et al. (2020), who similarly reported superior overall staining specificity and intensity of H&E over AB-PAS, Rapid PAP, and Gram stain, with AB-PAS being the best-performing special stain in their series.¹² In contrast, other authors evaluating AB-PAS, Ayoub-Shklar, and modified PAP stains have found the modified PAP or Ayoub-Shklar methods to outperform AB-PAS for staining intensity and specificity, suggesting that the relative performance of special stains may vary with staining protocol, tissue fixation, and observer scoring criteria.^{13,14} Ramulu et al. similarly found modified PAP stain comparable to Ayoub-Shklar and H&E stain for keratin demonstration in paraffin-embedded sections, supporting the broader premise that several economical special stains can serve as useful adjuncts to H&E.¹⁵

Taken together, these observations suggest that while H&E remains the practical gold standard for routine keratin assessment owing to its universal availability and diagnostic familiarity, AB-PAS can serve as a useful, low-cost adjunct special stain — particularly in settings where immunohistochemistry is not feasible — to confirm and better delineate keratinization patterns in well-differentiated OSCC. [Note to author: expand this section with your own interpretation once the exact statistical results and group-wise findings are inserted.]

VII. CONCLUSION

All four staining procedures — H&E, AB-PAS, Rapid PAP, and Gram stain — were able to distinctly demonstrate keratin in well-differentiated OSCC. Among the special stains, AB-PAS showed the best staining specificity, with intensity comparable to that of H&E, suggesting that AB-PAS may be used as an economical alternative special stain for the visualization of keratin. Nevertheless, on an overall comparative basis, H&E continues to remain the gold standard for the demonstration of keratin in well-differentiated OSCC.

ACKNOWLEDGEMENTS

The authors thank the Department of Oral and Maxillofacial Pathology and Microbiology, Maharana Pratap College of Dentistry and Research Centre, Gwalior, for providing the archival material and laboratory facilities for this study. [Insert names of any technical staff or co-guides to be acknowledged.]

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

SOURCE OF FUNDING

Nil / Self-funded. [Confirm and edit as applicable.]

REFERENCES

- [1]. Moll R, Divo M, Langbein L. The human keratins: biology and pathology. *Histochem Cell Biol.* 2008;129(6):705–33.
- [2]. Schweizer J, Winter H. Keratin biosynthesis in normal mouse epithelia and in squamous cell carcinomas. *J Biol Chem.* 1983;258:13268–72.
- [3]. Nagle RB, McDaniel KM, Clark VA, Payne CM. The use of antikeratin antibodies in the diagnosis of human neoplasms. *Am J Clin Pathol.* 1983;79:458–66.
- [4]. Rao RS, Patil S, Majumdar B, Oswal RG. Comparison of special stains for keratin with routine H&E stain. *J Int Oral Health.* 2015;7(4):1–5.
- [5]. Srivastava A, Singh A, Sah K, Raj V, Gupta B. Comparison of modified Kreyberg's, modified Papanicolaou, Ayoub-Shklar and Hematoxylin and Eosin stain to demonstrate keratin in paraffin embedded tissue sections. *J Dent Spec.* 2017;5:131–47.
- [6]. Akhter M, Hossain S, Rahman QB, Molla MR. A study on histological grading of oral squamous cell carcinoma and its co-relationship with regional metastasis. *J Oral Maxillofac Pathol.* 2011;15:168–76.
- [7]. World Health Organization. Oral health [Internet]. Geneva: WHO; [cited 2026 Sep 13]. Available from: <https://www.who.int/news-room/fact-sheets/detail/oral-health>
- [8]. Schweizer J, Winter H. Keratin biosynthesis in normal mouse epithelia and in squamous cell carcinomas. mRNA-dependent alterations of the primary structure of distinct keratin subunits in tumors. *J Biol Chem.* 1983;258:13268–72.
- [9]. Suvana SK, Layton C, Bancroft JD, editors. *Bancroft's Theory and Practice of Histological Techniques*. 7th ed. Edinburgh: Churchill Livingstone Elsevier; 2013.
- [10]. Johnson PL, Klein MN. Application of Papanicolaou stain to paraffin section. *Stain Technol.* 1956;5:223–5.
- [11]. Patil DB. A short study of keratin demonstration potential of Giemsa stain, Gram's stain and Haematoxylin-Eosin stain in paraffin embedded tissue sections. *Int J Sci Res.* 2018;7:2277–9.
- [12]. Bala J, Gupta U, Saxena S, Sankhla B, Sireesha SK, Bhargava A, et al. Comparative study of special and Hematoxylin and Eosin stains in oral squamous cell carcinoma for keratin. *Int J Sci Res.* 2020;9:2277–9.
- [13]. Jyoti R, Wadhwan V, Sharma P, Yadav L. Qualitative analysis of keratin in oral squamous cell carcinoma using Alcian Blue–Periodic Acid Schiff, Ayoub-Shklar, and modified Papanicolaou as special stains. *J Oral Maxillofac Pathol.* 2025;29(1):61–5.
- [14]. Anthwal N, Gupta S, Singh RP, Tyagi N, Gupta H. Comparison of Ayoub-Shklar stain, Dane-Herman, modified PAP and routine Hematoxylin and Eosin stain for the keratin identification. *Int Arch Integr Med.* 2020;7:1–8.
- [15]. Ramulu S, Kale AD, Hallikerimath S, Kotrashetti V. Comparing modified PAP stain with Ayoub-Shklar and H&E stain for demonstration of keratin in paraffin embedded tissue sections. *J Oral Maxillofac Pathol.* 2013;17:23–30.



Figure 1: Paraffin embedded blocks



Figure 2: Microtome (automatic)



Figure 3: Wax block holder for microtome



Figure 4: Slide warmer



Figure 5: Water bath(46 degree centigrade)



Figure 6: Binocular microscope



Figure 7: Rapid Papanicolaous (Rapid PAP)



Figure 8: Alcian Blue- Periodic Acid Schiff (AB-PAS)



Figure 9: H & E stains