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RESEARCH ARTICLE
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A CLINICOPATHOLOGICAL STUDY FOR EARLY DIAGNOSIS OF ORAL PRECANCEROUS AND CANCEROUS LESIONS USING TOLUIDINE BLUE AND CONFIRMING WITH HISTOPATHOLOGICAL EXAMINATION

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Abstract

Background: Oral cancer, the sixth most common malignancy globally, has one of the lowest 5-year survival rates, primarily due to delayed diagnosis. Early detection and intervention are critical for improving survival, yet current diagnostic methods often fall short. This study aimed to evaluate the effectiveness of a 1% Toluidine blue solution for detecting dysplastic oral lesions and to compare its results with histopathological findings.

Methods: A prospective analytical study was conducted with 100 patients presenting with symptoms of oral premalignant or malignant lesions at the Department of Otorhinolaryngology, Index Medical College Hospital and Research Centre, Indore. Each patient underwent Toluidine blue staining followed by biopsy for histopathological evaluation. The diagnostic performance of Toluidine blue was assessed for sensitivity, specificity, PPV and NPV.

Results: The mean age of participants was 45.55 ± 12.08 years, with a predominance of males (71.0%) and rural residents (77.0%). Lesions were most frequently located on the buccal mucosa (36.0%) and the lateral border of the tongue (35.0%). Toluidine blue staining was positive in 74% of cases. The sensitivity of Toluidine blue staining for detecting precancerous or cancerous lesions was 80.6%, with a specificity of 75.0%. The PPV was 95.9%, and the NPV was 34.6%. A Cohen's kappa

value of 0.370 ($p < 0.05$) indicated fair agreement between Toluidine blue staining results and histopathological diagnoses.
Conclusion: Toluidine blue staining is a simple, non-invasive method effective in detecting high-risk oral lesions and aiding early oral cancer diagnosis. Further research is needed to enhance its diagnostic accuracy and evaluate its use in various healthcare settings.

Keywords: Oral cancer, Toluidine blue staining, Early detection, Histopathology, Diagnostic tools

INTRODUCTION

"It is hard to look at the tumour and not come away with the feeling that one has encountered a powerful monster in its infancy" (Mukherjee, 2010).

Oral cancer ranks as the 6th most prevalent cancer worldwide. [1] Its incidence is particularly high in Southern Asia, including India and Sri Lanka, where it is a leading cause of death for both men and women. [2] Nearly 50% of oral cancer cases are diagnosed at an advanced stage, with a 5-year survival rate varying between 20-40% depending on the tumor's location.[3] Squamous cell carcinoma (OSCC) constitutes 92-95% of oral cancer cases.[4] The likelihood of developing oral cancer increases with age, primarily affecting those over 50, although cases in individuals under 40 have also been documented.[1]

OSCC frequently arises from oral potentially malignant disorders (OPMDs) such as leukoplakia (LPA), oral submucous fibrosis (OSF), and erythroplakia.[5] Timely detection of OPMDs and OSCC is crucial for enhancing survival rates and quality of life. Diagnostic delays can be attributed to both patient and physician factors, including the necessity for scalpel biopsy for confirmation and the lack of effective non-invasive diagnostic tools. Early detection is vital for prompt treatment and improved prognosis.[6]

Conventional oral examination (COE) is the standard approach for identifying OPMDs and OSCC, with biopsy confirming the diagnosis by revealing cellular and tissue-level changes.[7] Additional diagnostic methods include Toluidine blue staining, oral brush biopsy, and scalpel biopsy with histopathology.[8] Although histopathology is considered the gold standard, it is invasive, time-consuming, and costly. Emerging techniques, such as optical diagnosis, DNA marker analysis, and biomarkers in biofluids, offer potential but also come with limitations.[5]

Several methods have been introduced to enhance clinical examinations for the early detection of oral and oropharyngeal cancers. These techniques include toluidine blue staining, autofluorescence, veloscope, acetic acid staining, chemiluminescence, and oral exfoliative cytology.[6]

Toluidine blue, an acidophilic metachromatic dye, is used to detect early oro-pharyngeal premalignant and malignant lesions. It selectively stains acidic tissue components like DNA and RNA, which are more prevalent in dysplastic and malignant tissues compared to normal epithelium. Toluidine blue binds to nucleic acids, targeting tissues with high DNA and RNA content, and is partially soluble in water and alcohol. [9-13]

Healthy oral mucosa typically does not stain, though the dye may be retained in areas such as tooth fissures and tongue papillae, causing weak staining in other parts of the oral cavity.[14] To distinguish true suspicious areas from weakly stained regions caused by saliva or other factors, acetic acid swabbing can be employed. Although inflammatory and ulcerative lesions might also retain the dye and result in false positives, this issue can be mitigated by re-evaluating the lesions after addressing potential causes, as inflammatory changes typically resolve within two weeks. [15]

Over the past 30 years, studies utilizing toluidine blue to detect dysplastic and malignant oral mucosal lesions have yielded inconsistent results, leading to ongoing debate regarding its effectiveness. Furthermore, there is a lack of research specifically addressing toluidine blue staining for oral cancer in India. This underscores the importance of our study, which aimed to evaluate the diagnostic efficacy of a 1% solution of modified toluidine blue in identifying dysplastic changes in oral mucosal lesions and to compare its results with histopathological examination.

MATERIAL AND METHODS

The present prospective analytical study was conducted at the Department of Otorhinolaryngology, Index Medical College Hospital and Research Centre, Indore. It involved 100 patients of all age groups from the ENT OPD who exhibited symptoms of oral premalignant lesions or suspected oral malignancy. Ethical approval was obtained from the institutional ethics committee, and written informed consent was secured from all participants.

Inclusion Criteria

- Individual of all age group and gender presenting with signs or symptoms suggestive of suspected oral malignancies coming to the Department of ENT, in our institute.
- Patients who consented for participation in the study.

Exclusion Criteria

- Patients having suspected allergy to dyes and such chemicals.
- Patients who are already on treatment for oral malignancies.

Methodology

For the study, detailed histories and epidemiological data, including age, disease duration, and exposure to potential carcinogens, were collected from subjects with oral and oropharyngeal lesions using a questionnaire, following informed consent. Each patient underwent a comprehensive head and neck examination and those found to have symptoms and signs suggestive of oral premalignant lesions and suspected oral malignancy were further subjected to staining with a 1% solution of modified toluidine blue. Routine blood investigations were done. All identified lesions were biopsied under local anesthesia using punch or wedge biopsy techniques and subsequently sent for histopathological evaluation.

Preparation of Mouth Rinses and Staining Protocol [16]

The technique of application involved rinsing of the mouth with water for 20 s to remove debris. Followed by application of 1% acetic acid for 20 s to remove ropey saliva. Then 1% toluidine blue oral rinse was given for 20 s. Again 1% acetic acid rinse was performed to remove mechanically retained stain. Finally, the mouth was rinsed with water.

Lesions that exhibited dark blue staining were deemed positive for premalignant or malignant tissue, while those with light staining or no color were considered negative and were scheduled for follow-up. If the lesions did not resolve, they were biopsied. Informed consent was obtained from patients prior to biopsy, which was performed under local anesthesia. The pathologists who examined the biopsies were not informed about the clinical or staining evaluations of the samples.

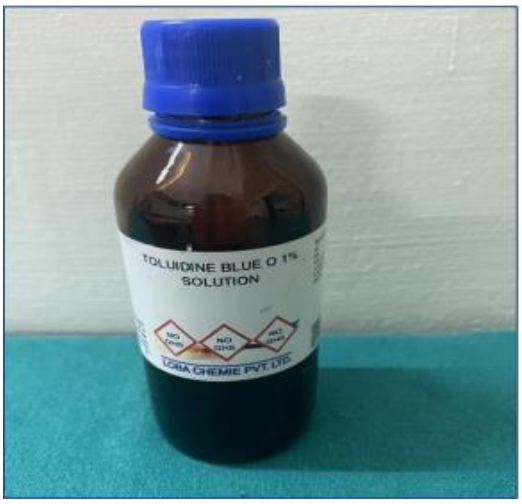


Figure 1. Toluidine blue 0.1% solution for staining

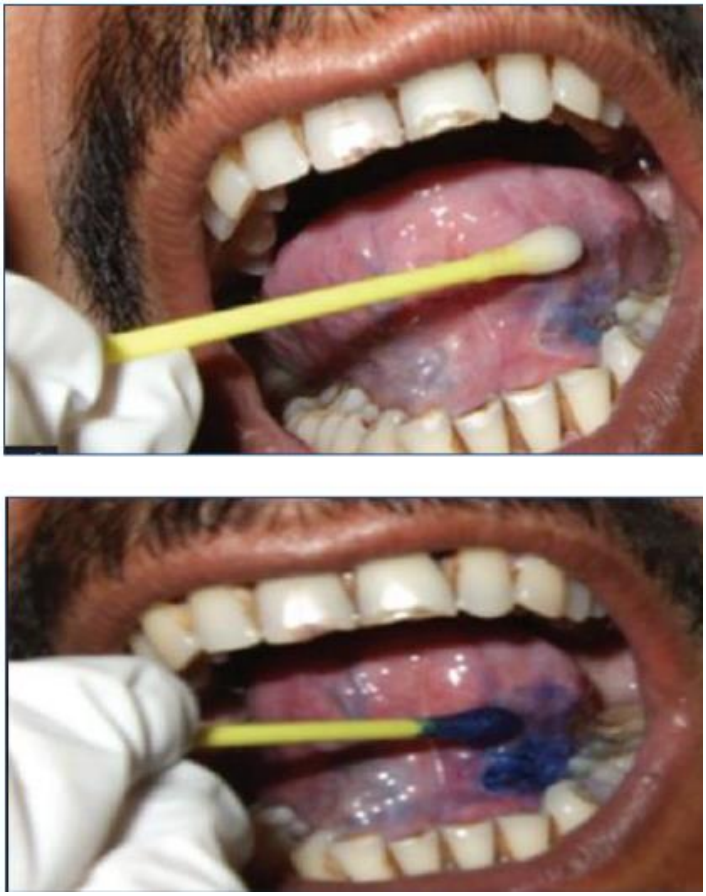


Figure 2. Staining for oral lesions using Toluidine blue 0.1% solution

Statistical Analysis

The raw data was entered and analyzed using Microsoft Excel 2010 and SPSS 20.0 for Windows. Prevalence rates were calculated with 95% confidence limits, and a descriptive analysis of the population was conducted. Categorical variables were expressed in absolute values and percentages, with comparisons made using the Pearson test. Continuous variables with a normal distribution were described as mean ± SD.

The diagnostic performance of 1% toluidine was assessed using statistical tests such as sensitivity, specificity, PPV, NPV, and accuracy, with Histopathological Examination (HPE) serving as the gold standard. Correlations between quantitative variables were evaluated using Pearson's or Spearman's coefficient of correlation, and associations between variables were determined by the Chi-Square test. A p-value of less than 0.05 was considered statistically significant.

RESULTS

The study involved 100 subjects with benign, precancerous, and cancerous lesions, with a mean age of 45.55 ± 12.08 years. The majority of subjects (34.0%) were in the 31-40 year age group, and over 80% were within this range. There was a higher prevalence of male subjects (71.0%) compared to females (29.0%). Most participants (77.0%) were from rural areas.

All subjects reported oral ulcers, with additional common complaints including inadequate mouth opening (30.0%) and pain on swallowing (28.0%). The average duration of symptoms was 7.09 ± 6.13 months. Lesions were most frequently observed on the buccal mucosa (36.0%) and the lateral border of the tongue (35.0%). Mean Size of the lesion was 3.1200 $\pm$ 1.53925. Clinical diagnoses included carcinoma of the tongue in 37.0% of subjects and leukoplakia in 29.0%. Among benign lesions, mucocele was the most common diagnosis (10.0%).

Toluidine blue testing was positive in 74% of subjects. Statistical analysis showed a significant association between lesion type and Toluidine blue staining results (Chi-square value = 46.894, df =

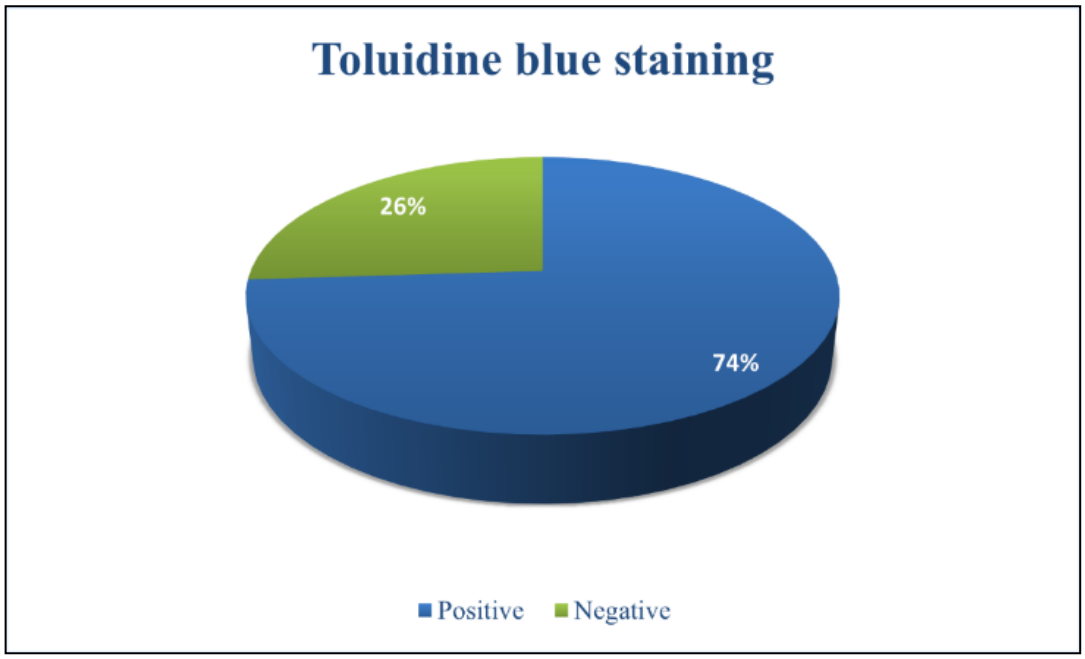
12, $p < .001$). The Cohen's kappa value of 0.370 ($p < .05$) indicated a fair agreement between Toluidine blue staining results and clinical examination and histopathological diagnoses.

Parameter	Frequency (N=100)	Percentage
Age groups		
20 years or less	2	2.0
21-30 years	6	6.0
31-40 years	34	34.0
41-50 years	27	27.0
51-60 years	22	22.0
61-70 years	7	7.0
71-80 years	1	1.0
81-90 years	1	1.0
Gender		
Male	71	71.0
Female	29	29.0
Residence		
Urban	23	23.0
Rural	77	77.0
Adverse habits		
Smokeless tobacco	87	87.0
Alcohol	37	37.0
Betel nut	56	56.0
Smoking tobacco	39	39.0
Chief complaints		
Oral ulcer	100	100.0
Inadequate mouth opening	30	30.0
Difficulty in swallowing	9	9.0
Pain on swallowing	28	28.0
Change in voice	5	5.0
Others (swelling)	3	3.0
Site of lesion		
Gingivolabial sulcus	2	2.0
Lateral border of tongue	35	35.0
Buccal mucosa	36	36.0
Retromolar trigone	5	5.0
Lower alveolus	2	2.0
Inner surface of lower lip	9	9.0
Gingivobuccal sulcus	2	2.0
Posterior 1/3rd of tongue	4	4.0
Dorsum of tongue	2	2.0
Vermillion border of lip	1	1.0
Lower gingiva	2	2.0

Table 1. Clinicodemographic profile of patients

Provisional diagnosis	Frequency	Percentage
Leukoplakia	29	29.0
Oral submucous fibrosis	3	3.0
Pemphigus vulgaris	2	2.0
Mucocele	10	10.0
Carcinoma tongue	37	37.0
Carcinoma buccal mucosa	13	13.0
Carcinoma alveolus	3	3.0
Carcinoma retromolar trigone	3	3.0

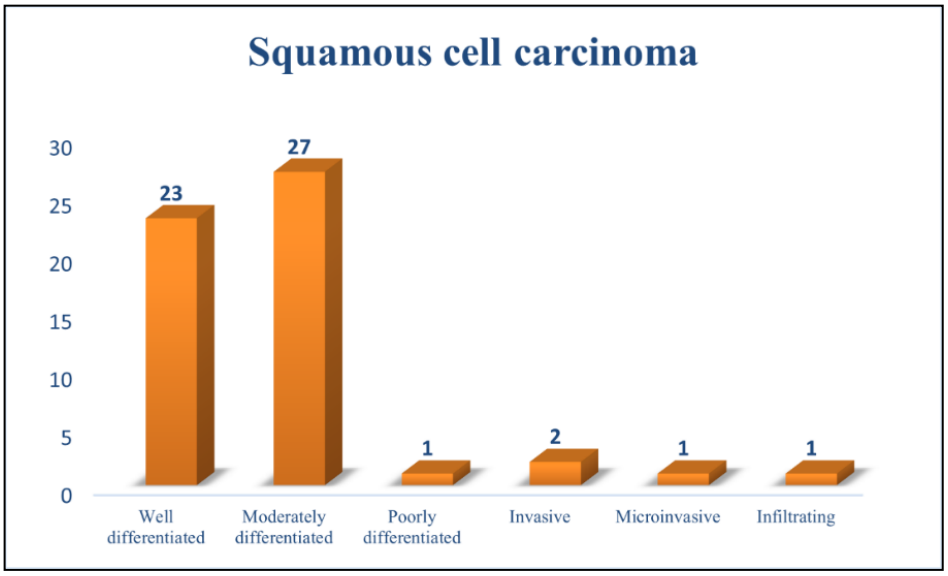
Table 2. Distribution of subjects based on provisional diagnosis



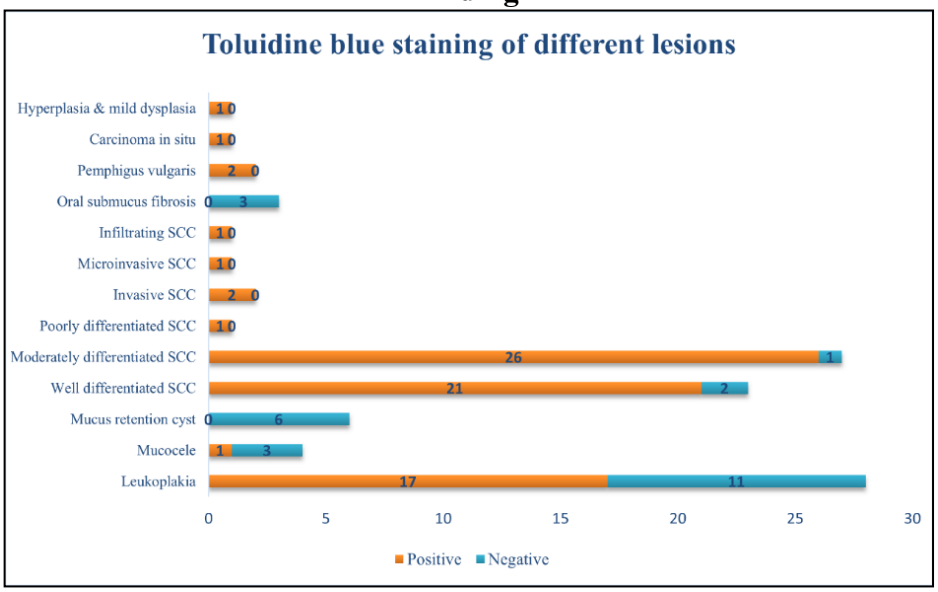
Graph 1. Distribution of subjects based on Toluidine blue staining

Histopathological diagnosis		Frequency	Percentage
Leukoplakia		28	28.0
Mucocele		10	10.0
Oral submucous fibrosis		3	3.0
Pemphigus vulgaris		2	2.0
Hyperplasia and mild dysplasia		1	1.0
Carcinoma *in situ*		1	1.0
Squamous cell carcinoma	Well differentiated	23	23.0
	Moderately differentiated	27	27.0
	Poorly differentiated	1	1.0
	Invasive	2	2.0
	Microinvasive	1	1.0
	Infiltrating	1	1.0

Table 3. Distribution of subjects based on histopathological diagnosis



Graph 2. Distribution of subjects with squamous cell carcinoma based on histopathological finding



Graph 3. Comparison of results of Toluidine blue staining amongst different benign lesions and pre-cancerous or cancerous lesion

			Clinical examination		Total
			Pre-cancer/cancer (Positive)	**Benign (Negative)**	
Toluidine blue Staining	Positive	Frequency	71	3	74
		Percentage	80.7%	25.0%	74.0%
	Negative	Frequency	17	9	26
		Percentage	19.3%	75.0%	26.0%
Total		Frequency	88	12	100
		Percentage	100.0%	100.0%	100.0%

*Kappa value- 0.370, p value- .001; revealed statistically significant fair agreement between the Toluidine blue staining results and histopathological diagnosis

Table 4. Correlation between findings of Toluidine blue staining and findings of clinical examination.

			Histopathology		Total
			Pre-cancer/cancer (Positive)	Benign (Negative)	
Toluidine blue Staining	**Positive 74%**	Frequency	71	3	74
		Percentage	80.7%	25.0%	74.0%
	Negative 26%	Frequency	17	9	26
		Percentage	19.3%	75.0%	26.0%
Total		Frequency	**88**	**12**	**100**
		Percentage	**100.0%**	**100.0%**	**100.0%**

*Kappa value- 0.370, p value- .001; revealed statistically significant fair agreement between the Toluidine blue staining results and histopathological diagnosis.

Table 5. Correlation between findings of Toluidine blue staining and findings of histopathological examination.

DISCUSSION

Prevention, early detection, and prompt referral are crucial for the effective treatment of cancer, offering the best chance for a cure. Recent advancements have introduced vital dyes and stains, such as Crystal violet, Lugol's iodine, and toluidine blue, as valuable diagnostic tools for identifying early potentially malignant and malignant lesions. [17] These methods are simple, non-invasive, inexpensive, and can be performed as outpatient procedures with minimal delay.

Toluidine blue was first reported in 1949 in the Journal of the American Medical Association (JAMA) [18] and has since been recognized for its ability to selectively stain acidic tissue components like DNA and RNA. This staining technique is effective in detecting early nuclear changes associated with malignancy.

In the study involving 100 patients with benign, precancerous, and cancerous oral lesions, the mean age was 45.55±12.076 years, with the majority (34.0%) falling into the 31-40 year age group. Over 80% of the participants were between 31 and 40 years old, which is somewhat younger compared to the 55.3±16.1 years reported by Cancela-Rodríguez et al. [19]

In terms of gender distribution, there was a notable male predominance (71.0%), which aligns with findings of study done by Pallagatti et al. [20] from Haryana, India, where 90.62% of the patients were male.

Lesions were most commonly found on the buccal mucosa (36.0%) and the lateral border of the tongue (35.0%). This is consistent with study done by Pallagatti et al. [20] who reported that 57.5% buccal mucosa lesions among 32 patients and Crăcană et al.'s [21] observation of lip lesions in 32.67% of cases.

The Toluidine blue test proved positive in 74% of the subjects in our study, which is higher than the 69.5% positivity reported by Pallagatti et al. [20] and the 70.3% by Warnakulasuriya WA et al [22]. Allegra et al. [23] found a lower positivity rate of 57.7% among 45 patients.

Statistical analysis showed a Cohen kappa value of 0.370 with a p-value <0.05, indicating a fair agreement between Toluidine blue staining results and histopathological diagnosis. The study demonstrated Toluidine blue staining's sensitivity at 80.6% for detecting precancerous or cancerous lesions, with a specificity of 75.0%. The positive predictive value (PPV) was 95.9%, and the negative predictive value (NPV) was 34.6%. This implies that Toluidine blue staining was effective in identifying 80.6% of lesions as precancerous or cancerous but had limitations in accurately ruling out non-cancerous conditions.

Comparatively, Vijaykumar V et al. [6] reported a sensitivity of 92.6% and specificity of 67.9%. Cancela-Rodríguez et al. [19] reported lower sensitivity (65.5%) and specificity (73.3%) with Toluidine blue, while Allegra et al. [23] achieved high sensitivity (96.2%) but lower specificity (77.7%). Onofre et al. [24] reported a sensitivity of 77% and specificity of 67%, whereas Pallagatti et al. [20] found

sensitivity and specificity of 95% and 71.4%, respectively. Vashisht et al. [25] also observed high sensitivity (86.36%) and specificity (76.9%). These variations highlight the differing effectiveness of Toluidine blue across studies, underscoring the need for further research to refine its diagnostic accuracy.

A key limitation of our study is that, being hospital-based, it does not address the utility of the Toluidine blue dye test in primary care settings. This means the findings may not fully represent how effective the test could be when used in less specialized, primary healthcare environments.

CONCLUSION

A white patch or a persistent ulcer in the oral cavity may signal an oral malignancy, with red lesions indicating a more severe risk. In India, local tobacco use frequently contributes to the occurrence of oral premalignant lesions, predominantly affecting the buccal mucosa. The rural population often faces neglect within the public health system, leading to delays in seeking quality medical care, especially when symptoms are minor. This delay can exacerbate the challenges of distinguishing between benign and malignant lesions. Therefore, it is crucial to find a reliable and safe method for differentiation. In conclusion, Toluidine blue staining could serve as a valuable tool in identifying high-risk lesions and facilitating the early diagnosis of oral cancer.

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