Original Article

Application of *in vivo* stain of methylene blue as a diagnostic aid in the early detection and screening of oral cancerous and precancerous lesions

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Abstract

Background: Periodic clinical examination of the oral cavity is the mainstay for the early detection of oral cancers which can be further aided by screening individuals with high-risk factors that will identify candidates who should receive treatment to prevent cancer progression and reduce patient mortality. Among the diagnostic tools, *in vivo* staining is advocated as a simple, inexpensive and fairly sensitive method.

Materials and Methods: The present study involved the examination of fifty patients suspected of oral malignant or precancerous lesions by methylene blue staining. The results of methylene blue uptake were compared with a simultaneous biopsy of these lesions, while benign epithelial lesions were included as the negative subjects of screening.

Results: The results revealed a sensitivity of 89%, a specificity of 91%, a positive predictive value of 97% and a negative predictive value of 73%.

Conclusion: We recommend that methylene blue staining is a useful diagnostic adjunct in a large, community-based oral cancer screening program for high-risk individuals.

Keywords: Methylene blue, oral cancer, oral precancer, vital staining

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INTRODUCTION

Oral mucosal lesions have beleaguered the humankind since long. Globally, oral cancer constitutes one of the most common cancers, with a very high incidence in the developing countries. In the Indian scenario, oral cancer is the second most common cancer ranging from innocuous mucosal alterations needing simple therapeutic remedies and patient counseling to life-threatening lesions.[1]

Silverman reported that oral carcinoma in the early stage may appear as a small, apparently harmless area of induration or localized change such as erosion, erythema or keratosis. Because of the variability in signs and symptoms among oral cancer patients, even exceptional clinical judgment and extensive experience do not preclude diagnostic errors. Although apparently benign, any oral lesion that does not respond to the usual therapeutic measures should be considered malignant until histologically shown to be

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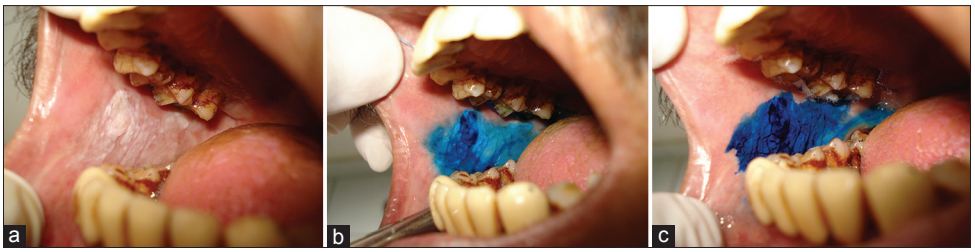


Figure 1: (a) Clinical photograph of homogenous leukoplakia (b) homogenous leukoplakia prerinse (c) homogenous leukoplakia postrinse

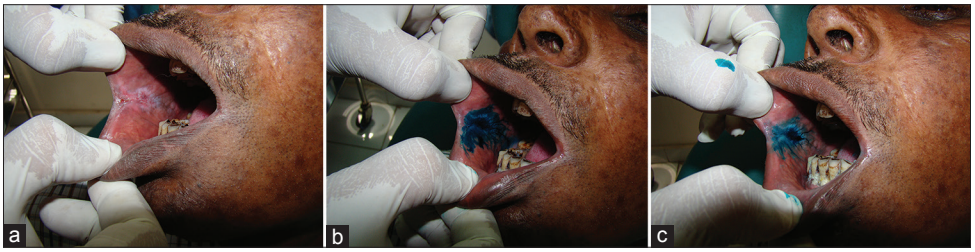


Figure 2: (a) Clinical photograph of nonhomogenous leukoplakia (b) nonhomogenous leukoplakia prerinse (c) nonhomogenous leukoplakia postrinse

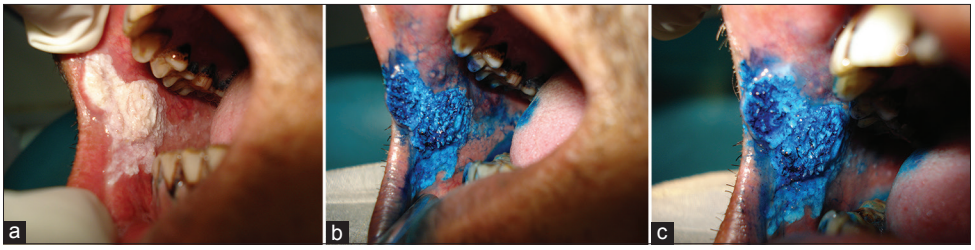


Figure 3: (a) Clinical photograph of verrucous leukoplakia (b) verrucous leukoplakia prerinse (c) verrucous leukoplakia postrinse

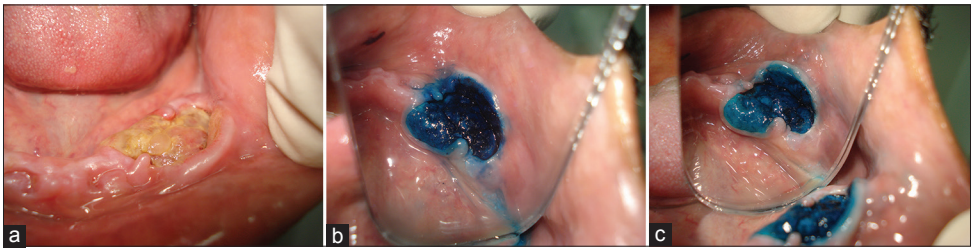


Figure 4: (a) Clinical photograph of malignant ulcer (b) malignant ulcer prerinse (c) malignant ulcer postrinse

benign. The gold standard for the definitive diagnosis of oral cancer is histologic examination of tissue from a biopsy.[2]

Oral cancer is generally preceded by some benign lesions for a varying length of time. Many of them show high cancerous potential, and therefore are termed as "precancerous." Even though only small proportion of precancerous progress to oral cancer, this development forms a source for over 70% of oral cancers in India. Individuals with precancer run a risk that is 69 times higher for them to develop oral cancer compared to tobacco users who are not precancerous. The recognition and management of precancer, therefore, constitutes a vital oral cancer control measure.[1]

By far, the most effective way of combating oral cancer is by early diagnosis followed by adequate treatment. The clinician's dilemma is differentiating cancerous lesions from a multitude of other ill-defined, controversial and poorly understood lesions that also occur in the oral cavity. Most oral lesions are benign, but may have an appearance that may be easily confused with a malignant lesion, and some are now considered premalignant because they have been statistically correlated with subsequent cancerous changes. Conversely, some malignant lesions seen in an early stage may be mistaken for a benign change.[2]

With the aim of improving the efficiency of this diagnosis, techniques are being developed to complement clinical

examination and to facilitate the identification of initial carcinomas. In addition, using adjunctive aids such as toluidine blue (also referred to as tolonium chloride) has been widely accepted to improve the effectiveness in large-scale screening for oral cancer diagnosis. However, toluidine blue is hazardous if swallowed. Methylene blue is another recently proposed dye, has physiochemical structure similar to toluidine blue with the added advantage of being less toxic to the human body and has recently been proposed for *in vivo* staining.[3,4]

At present, to the best of our knowledge, only one study has been reported in literature regarding the detection of oral cancer or precancer lesions by *in vivo* staining with methylene blue. The application of this material in detecting oral cancer or precancerous lesions has so far not been addressed further. The present study is an attempt to evaluate the sensitivity and reliability of *in vivo* staining with methylene blue as a diagnostic adjunct in the early detection and screening of oral cancer and precancerous lesions by dental professionals, in order to reduce the high mortality rate.

MATERIALS AND METHODS

This study was carried out in the Department of Oral Medicine and Radiology, Rama Dental College, Hospital and Research Centre, Kanpur (Uttar Pradesh) India, to evaluate the sensitivity and reliability of *in vivo* staining with methylene blue as a diagnostic adjunct in the early detection and screening of oral cancer and precancerous lesions. A total of fifty patients who fulfilled our study criteria (potentially malignant lesion) were selected from the dental outpatient department (OPD) and fifty dental students (control group) who volunteered for the study were randomly enrolled (with a mean age of 22 years) for the study. All the patients were evaluated with a dental history taking and thorough clinical examination pertaining to the mucosal lesions which were carried out with the following clinical diagnosis:

1. Homogeneous leukoplakia: White, uniform, flat lesion with a smooth, wrinkled or corrugated surface, not able to be scraped
2. Nonhomogeneous leukoplakia: White lesion with an irregular and exophytic surface
3. Erythroplakia: Red lesion with an ill-defined margin
4. Ulceration: Localized and superficial lesion that does not heal after local treatment.

Gargling solution

A set of methylene blue dye system was used which included the following two bottles of solution:

- Bottle A: Dye rinse solution containing active ingredient as methylene blue 1% with the addition of 1% malachite, 0.5% eosin, glycerol and dimethyl sulfoxide
- Bottle B: Pre- and post-rinse solution containing 1% lactic acid, raspberry flavoring ingredient and purified water.

Procedure for staining

All the study participants were instructed to rinse their mouth with bottle B solution for 20 s to remove food debris and excess saliva and to provide a consistent oral environment. The mucosa of the target area was gently dried with a gauze and power air spray to ensure that the lesion was not contaminated with saliva. After the target area is dried, 1% methylene blue dye (bottle A) was applied and let for 20 s. Patients then rinsed their mouth again with bottle B solution for 20 s to wash out the excess dye. The pattern of dye retention was assessed by the intensity of stain retention on the lesion. Local, stippled, patchy and deep blue stains were marked as positive reaction, whereas wide, shallow or faint blue stains were marked as negative reaction. For equivocal staining, bottle B solution was applied with cotton rolls to wipe out the staining surface. If the blue stain was washed out, negative reaction was recorded and *vice versa*. The results of methylene blue dye staining were recorded with photographs by following the standardized methods [Figures 1-4].

The incisional biopsy was performed simultaneously in the most obvious staining area of the suspicious lesion of all patients under local anesthesia. If there was no dye uptake in the lesions, the biopsy specimen was taken from the area judged by a specialist's experience. All the specimens were then fixed in 10% neutral buffered formalin and submitted for routine histopathologic diagnosis in the pathology laboratory.

Later, the specimens were microscopically evaluated by pathologists who were blind to the results of methylene blue stain. The pathology reports of the lesions were classified as: (1) Benign lesions – hyperkeratosis; (2) precancerous lesions including verrucous hyperplasia, dysplasia and (3) malignant lesions including verrucous carcinoma and squamous cell carcinoma.

In the control group, as methylene blue dye was not used to examine the oral cavity, it was necessary to verify that the dye is not retained on normal mucosa. The results demonstrated that there was no retained dye in the control group and the performance of biopsy in normal mucosa would not be ethical.

RESULTS

Data analysis

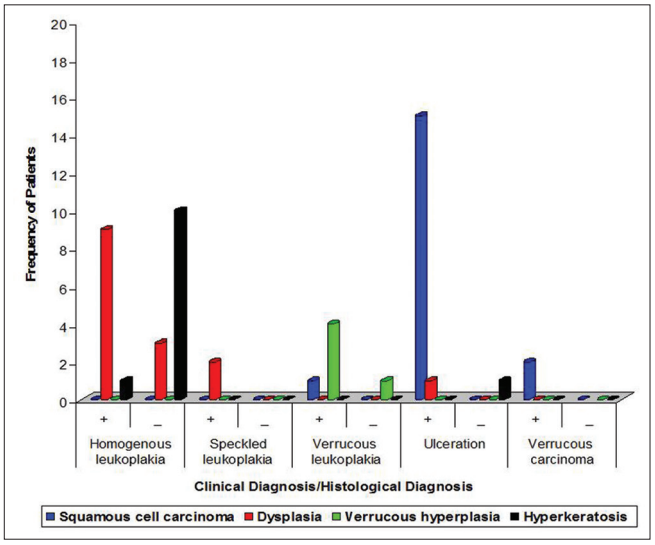
The pathologically proven cancerous and precancerous lesions were the targets of screening. The results of positive/negative uptake of methylene blue in each lesion were correlated with the histopathologic diagnosis. Statistical analysis was performed, including sensitivity, specificity and positive and negative predictive values. The association of methylene blue uptake and pathologic diagnosis among the precancerous/cancerous group, benign group and normal group was analyzed by using Chi-square test and χ^2 tab at 5% of level of significance.

Subject characteristics

The total study sample consisted of fifty patients each in case and control groups. The most common age group in the study sample involved between 31 and 50 years of age with 60% followed by 51 and 70 years of age with 22% and 21 and 30 years of age with 12%. The control group ranged from 22 to 24 years. The suspected lesions were distributed over the buccal mucosa (n = 29), labial mucosa (n = 2), tongue (n = 3), alveolar mucosa (n = 15) and floor of the mouth (n = 1). In the control group, as methylene blue dye was not used to examine the oral cavity, it was necessary to verify that the dye is not retained on normal mucosa. The results demonstrated that there was no retained dye in the control group and the performance of biopsy in normal mucosa would not be ethical.

The clinical and histopathologic diagnosis of oral lesions and the results of staining are shown in Table 1 and Graph 1. On statistically comparing the data, null hypothesis states that there is no association between the clinical and histologic diagnosis of cancer and precancerous lesions using the method of staining procedure. As the calculated value of χ^2 came out to be greater than its tabulated value, null hypothesis was rejected. The coefficient of contingency was equal to 0.6278 which is quite high. Because there is very high degree of association between histopathological and clinical diagnosis, this hypothesis shows that the methylene blue staining method employed in the detection of cancer and precancerous lesions is quite effective in the diagnosis of cancer and precancerous lesions.

Sensitivity represents the proportion of histologically proved cancer and precancerous lesions which are detected by positive methylene blue staining. In the current study, 34 of 38 pathologically proved cancer or precancerous lesions were positive with deep and focal methylene blue staining. The overall sensitivity was 89%. Among the four false-negative cases, three cases clinically presented as a homogeneous leukoplakia on the buccal mucosa with a pathologic diagnosis of epithelial dysplasia and one case clinically presented as a verrucous leukoplakia on the buccal mucosa with a pathologic diagnosis of verrucous hyperplasia. They were stained with a faint blue color. Specificity suggests the proportion of pathologic benign lesions, neither precancerous nor cancer lesions, which is



Graph 1: Clinical and histopathological diagnosis

Table 1: Clinical and histological diagnosis of cancer and precancer lesions with results of methylene blue staining

Histologic diagnosis	Clinical diagnosis										
	Homogenous leukoplakia		Speckled leukoplakia		Verrucous leukoplakia		Ulceration		Verrucous carcinoma		Total frequency (f_{io})
	+	–	+	–	+	–	+	–	+	–	
Cancer lesions											
Squamous cell carcinoma	0	0	0	0	1	0	15	0	2	0	18
Precancer lesions											
Dysplasia	9	3	2	0	0	0	1	0	0	0	15
Verrucous hyperplasia	0	0	0	0	4	1	0	0	0	0	5
Benign lesions											
Hyperkeratosis	1	10	0	0	0	0	0	1	0	0	12
Total frequency (f_{oi})	10	13	2	0	5	1	16	1	2	0	50

χ^2 tab=21.0260; χ^2 cal=84.3478 ; Q=0.6278

Table 2.Efficacy of methylene blue application in pathologically proved cancer or precancer lesion

Type of tissue	Positive	(%)	Negative	(%)
Cancer/ Precancer (*n*=38)	34	(89)*	4	11
Benign (*n*=12)	1	(9)	11	(91)†
Control (*n*=50)	0	(0)	50	100
Positive predictive value	34/35	(97)		
Negative predictive value			11/15	73

*Sensitivity; †specificity

correctly identified as negative staining by methylene blue. In our study, 11 of 12 benign lesions showed negative staining; thus, the specificity was 91%. The one falsepositive case which clinically presented as a homogeneous leukoplakia on the buccal mucosa was histopathologically diagnosed as hyperkeratosi [Table 2]. Overall, the positive predictive value was 97% (34/35) and the negative predictive value was 73% (11/15). The predictive value of a positive test refers to the probability that the lesion is present given that the test result is positive.

DISCUSSION

The burden of oral cancer is increasing worldwide despite advances in diagnosis and treatment. Chronic diseases such as cancer and other noncommunicable diseases are exponentially replacing communicable diseases in India and other developing countries.[5] Despite improved surgical approaches, vastly improved reconstruction techniques and advances in radiation and medical oncology, the single-most effective route for improving the long-term outcome of oral cancer is early diagnosis, coupled with appropriate treatment.[2,6]

Dentists need to focus on screening patients considered to be at high risk and to act proactively to prevent morbidity and mortality from the disease process and should not wait for lesions to become symptomatic. There is a correlation between early diagnosis and improved survival rate.[7]

A number of techniques have been developed to supplement clinical examination, thus improving the diagnosis of early oral malignancy. Exfoliative cytology has a false-negative rate of approximately 30%. Although flow cytometry is a useful guide to imminent malignant change, it is far from being a simple chairside investigation. Similarly, acridine binding is not widely available in the clinical setting.[8]

Several clinical studies have evaluated the efficiency of *in vivo* staining with toluidine blue in the detection of dysplasia and malignant lesions. This vital staining method was used at first in medicine for detecting cervical dysplasia and carcinoma *in situ* in the 1960s.[9] Various studies were carried out to determine the feasibility of using toluidine blue rinse as a diagnostic aid for screening and detecting oral cancer and precancerous lesions.[10-13]

However, toludine blue is unsafe if swallowed, and was shown to have toxicity to fibroblasts. The Material Data Safety Sheet indicates that toluidine blue is probably toxic by ingestion, and it is seldom used for detecting cancers in other parts of the human body. Methylene blue is another recently proposed dye, has a similar chemical structure and exhibits similar physicochemical properties to toluidine blue. It is less toxic to the human body and has recently been proposed for *in vivo* staining in endoscopic examination.[3,4] Various studies on methylene blue have also been reported recently in detecting some gastrointestinal abnormalities such as Barrett's esophagus,[14,15] gastric cancer[16] and bladder cancer.[17-19]

The application of *in vivo* methylene blue staining study in the early detection and screening of oral cancer and precancerous lesions in India has not been reported so far. The present study was aimed to evaluate the sensitivity and reliability of *in vivo* staining with methylene blue as a diagnostic adjunct and to help in the early detection of oral cancer by dental professionals, in order to reduce the high mortality rate. A total of fifty oral cancerous or precancerous patients who visited the dental OPD and fifty dental students (control group) who volunteered were selected for the study.

Among all the statistical values, sensitivity rate and false negatives are the most important in evaluating the efficacy of certain diagnostic tools for detecting abnormal lesions. In the present study, 34 of 38 pathology-proven cancer or precancerous lesions showed positive staining with deep and focal methylene blue dye. Among the four false-negative cases, three clinically presented as a homogeneous leukoplakia on the buccal mucosa with a pathologic diagnosis of epithelial dysplasia and one clinically presented as a verrucous leukoplakia on the buccal mucosa with a pathologic diagnosis of verrucous hyperplasia. They were stained with a faint blue color. Overall, a sensitivity rate of 89% and a false-negative rate of 11% were reported [Table 2].

These findings were in accordance with the study conducted by Chen *et al*. in 1992 wherein 26 of 29 pathology-proven precancerous/cancer lesions showed positive staining with deep and focal methylene blue dye, with a sensitivity of 90% and a false-negative rate of 10%. Compared to the 72%–100% sensitivity reported in

the previous studies for toluidine blue dye, these values indicated that using methylene blue dye for the detection of cancer or precancerous lesions is acceptable.[3,4] The false-negative rate of 11% encountered in our study is probably because of the ambiguous light blue stain which may be misinterpreted as negative, but the clinical suspicion of malignancy still needs further biopsy to prove the diagnosis pathologically.

The exact mechanism for the uptake of methylene blue dye in epithelial cells is still not very clear, but it resembles toluidine blue dye in its acidophilic characteristic and may penetrate into cells with an abnormal increase in nucleic acid, thus resulting in different uptake mechanisms between normal and highly dysplastic or malignant cells.[3,4] Canto *et al*. reported that methylene blue mucosal staining is a safe, inexpensive, reproducible and highly accurate method of diagnosing specialized columnar epithelium in Barrett's esophagus.[15] Chen *et al*. reported that methylene blue resembles toluidine blue dye in its acidophilic characteristic and may penetrate into cells with an abnormal increase in nucleic acid, thus resulting in different uptake between normal and highly dysplastic or malignant cells.[3,4] Canto *et al*. suggested that, although the exact mechanism for the uptake of methylene blue dye in epithelial cells is still not known, the current hypothesis is that the entry of stain into cells results from absorption across the epithelial cells. The decrease or lack of methylene blue stain in highly dysplastic or malignant cells may be due to the decreased number of goblet cells and the increase in the nuclear-to-cytoplasmic ratio with increasing dysplasia grade.[14] Gill *et al*. suggested that the abnormal membranes of the lesions allow the cationic dye methylene blue to gain access and bind to the negatively charged nucleic acids in the nuclei.[18] Canto *et al*. reported that methylene blue staining improves the ability to diagnose dysplasia and cancer by giving distinct staining appearances between nondysplastic and dysplastic cells, particularly when dysplasia is severe.[14] Fukui *et al*. reported that the intensity of the stain was well correlated with histologic picture. In low-grade tumors, close cohesiveness of tumor cells impairs the penetration of the dye into the tumor tissue, resulting in a poor stain. Conversely, in high-grade tumors, the loss of surface cells and decreased cellular cohesiveness allow the penetration of the dye into deeper layers of tumor tissue and many tumor cells with hyperchromatic nuclei are stained. Furthermore, necrosis of superficial tumor tissue and inflammatory exudates such as fibrin debris and white blood cells that frequently are found, leading to an overall deep blue stain of these tumors. Simple inflammatory mucosa without atypia occasionally took a light stain.[17,20,21] Richart reported that, in the initial staining period, after the excess stain is removed, the area of blue-stained epithelium on the portion will contain not only the neoplastic epithelium but also false-positive areas, such as columnar epithelium, mucus or the intense inflammatory infiltrate associated with erosion. Areas of the epithelium which are highly cellular, but not neoplastic, also stain blue, but these areas of neoplasia, and blend into the adjacent epithelium gradually, whereas the areas of neoplasia tend to be sharply demarcated and to stain more intensely and uniformly. Because the intensity of the stain is correlated with nuclear density, the severity of the neoplastic process can be gauged roughly from the shade of blue, which ranges from a pale royal blue in minimal dysplasia, to a very intense royal blue in carcinoma *in situ*.[9,22]

The higher falsepositive rate encountered in their study may be related to the fact that the inflamed mucosa and traumatized area will retain dye stain.[3] Moreover, the less percentage of false positivity in our study means that less patients received biopsies. Nevertheless, rational management for patients with suspected oral lesions who have either a positive or negative methylene blue stain remains biopsy of the lesion.[23,24]

This method can be applied to screen highrisk patients with the habits of betel quid chewing or smoking which may include a large group of individuals with obvious oral lesions and those with normal oral mucosa.[23,25] To study these people and to reevaluate the efficacy of methylene blue stain in detecting oral cancerous or precancerous lesions, a large proportion of people with normal oral mucosa will lower the rate of false positives and result in higher specificity. Although we had a control group with normal oral mucosa, there was a flaw in the experimental design that these students had no habits of betel quid chewing and histories of smoking. However, individuals who had these habits without lesions were also not suitable to be our control group because the performance of biopsy in normal mucosa would not be ethical.

CONCLUSION

This is a preliminary study for the evaluation of sensitivity and reliability of *in vivo* staining with methylene blue as a diagnostic adjunct in the early detection and screening of oral cancerous and precancerous lesions. Toludine blue is widely used, but methylene blue staining is also used recently for the early detection of oral cancerous and precancerous lesions. In this study, methylene blue application was employed to evaluate various malignant and potentially malignant epithelial lesions and superficial

ulceration suspicious of malignancy. The results of this study conclude that methylene blue staining has nearly 89% sensitivity in detecting oral cancerous or precancerous lesions. Considering its low toxicity and the fact that it is cost-effective than toluidine blue, it may be convenient to substitute it for toluidine blue in large-scale oral screening of high-risk patients. Nevertheless, the pathology report from biopsy is still the gold standard to accurately diagnose the lesion before a treatment modality is determined.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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