ACTA SCIENTIFIC DENTAL SCIENCES (ISSN: 2581-4893)

Volume 2 Issue 11 November 2018

Research Article

Diagnostic Efficacy of 1% Methylene Blue Vital Staining in Individuals Suspected with Oral Potentially Malignant Disorders

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Received: September 12, 2018; **Published:** October 15, 2018

Abstract

Background: Oral cancer is one of the most common neoplasms worldwide with a high mortality rate in spite of advanced treatment modalities, which is attributed mainly to delayed diagnosis. It involves a multi-stage process of changes i.e. from normal to dysplasia, as seen in oral potentially malignant disorders (OPMDs) to eventually, malignancy. Hence, early diagnosis, at the potentially malignant level, can prove beneficial. Methylene Blue (MB) 1%dye, which is otherwise efficacious in detection of cancers of the bladder, stomach etceteraetera, is one such recent diagnostic tool. It is fairly sensitive, simple to use and with favorable properties similar to Toluidine Blue (TB), otherwise routinely used for oral cancer/OPMD screening. Methylene blue is cheaper and less toxic than TB, which is said to be toxic to fibroblasts and has a probability of inducing mutagenesis especially if the vitally stained cells are exposed to high energy irradiation like light. However, the use of MB in oral cancer/OPMD screening has not been documented as largely as TB, in the literature.

Aim: To evaluate the efficacy and diagnostic reliability of 1 % MB in-vivo staining in the early detection of OPMDs.

Methods: A total of 50 cases of both sexes (no specific age range) with clinically diagnosed OPMDs, who visited the outpatient department of Oral Medicine and Radiology, Saraswati Dental College and Hospital, Lucknow (India), were included in the study. Vital staining of the lesions with 1% MB dye was followed by biopsy from the site with maximum dye retention (or based on the clinician's judgement in clinically suspicious cases with no dye retention); the tissue specimens thus obtained were subjected to histopathological examination (gold standard) for deriving the final diagnosis. The results of histopathological examination and chair-side vital staining were compared subsequently. The inter-reliability Kappa statistic test was used to assess the association of MB uptake and histopathologic diagnosis among the OPMDs.

Results: The results revealed a sensitivity of 71.4%, specificity of 62.5%, positive predictive value of 90.9% and negative predictive value of 29.4%. The overall diagnostic accuracy of this technique was found to be 70% and the p-value showed equivocal significance ($p = 0.063$).

Conclusion: We conclude that the MB dye is an efficacious diagnostic adjunct and a suitable alternative for the routinely used TB dye, for large-scale, community-based OPMD and oral cancer screening programs among high-risk individuals.

Keywords: Methylene Blue; Oral Potentially Malignant Disorders; Oral Cancer; Screening; Vital Staining

Abbreviations

PMD: Potentially Malignant Disorders; TB: Toluidine Blue; LI: Lugol's Iodine; AA: Acetic Acid; MB: Methylene Blue; DNA: Deoxyribonucleic Acid; WHO: World Health Organization; PPV: Positive Predictive Value; NPV: Negative Predictive Value; p-value: Probability Value

Introduction

Oral Cancer is among the most common neoplasms in the world. Most cases are sequelae of the premalignant lesions and conditions of the oral mucosa, such as leukoplakia, erythroplakia, palatal lesion of reverse cigar smoking, oral submucous fibrosis, discoid lupus erythematosus, dyskeratosis congenita and epider-

molysis bullosa, which are now referred to as Potentially Malignant Disorders (PMDs), a terminology coined at the workshop by the WHO Collaborating Centre for Oral Cancer and Precancer held at UK in 2007 [1,2]. In spite of numerous advances in treatment modalities, the 5-year survival rate of the affected individuals still remains between 50 - 60% [3]. This is due to the inability of malignancy to be diagnosed at the initial stage itself. The early detection of oral PMDs by periodic clinical examination is thus essential to facilitate improved survival rates [1,4]. Numerous screening and diagnostic techniques have been formulated for the same, among which in vivo staining with the help of dyes is a relatively simple, inexpensive and fairly sensitive method, helpful in lesion margins and biopsy site selection [5,6]. The dyes routinely used for the same are Toluidine Blue (TB), Lugol's Iodine (LI) dyes and Acetic Acid (AA) [6,7]. The Methylene blue (MB) dye, which is usually used in screening of bladder, gastric and prostate cancers and Barrett's oesophagus, has now been found efficacious in the detection of oral cancer and oral PMDs [1,4,8]. It is similar to toluidine blue dye in numerous ways such as its acidophilic character and resultant strong attraction to acids including DNA depicted by deep blue colour clinically and significant sensitivity. Also, MB was shown to be relatively less toxic and cheaper in cost. Thus, even MB may be similarly employed for large scale screening of oral cancer and oral PMD victims [1,4]. Also, till now, only three studies proving the utility of MB for detection of oral PMDs and cancer are present in literature.

In the present study an attempt has been made to decipher the efficacy of MB as an alternative screening tool to facilitate early diagnosis of potentially malignant oral disorders in suspected cases and prevent full-fledged malignant transformation.

Materials and Methods

Subjects

After approval from the Institutional Human Ethical Committee and after signing the informed consent forms, a total of 50 patients of both sexes (no specific age range), visiting the outpatient department of Oral Medicine and Radiology between December 1, 2012 and June 30, 2014, with clinically suspicious potentially malignant oral disorders like Leukoplakia, Erythroplakia and oral Lichen Planus, were included in the study. Patients with history of oral cancer and surgery, having areas of inflammation or trauma intraorally, with abnormal blood parameters (showing pathologic changes), those suffering from psychiatric disorders, pregnant or lactating patients and those with lesions on the hard palate and gingival, were excluded from the study.

Dye System

The set of methylene blue dye system includes 2 bottles of solutions, in which Bottle A contains active ingredient methylene blue 1%, 1% malachite, 0.5% eosin, glycerol, and dimethyl sulfoxide and Bottle B contains 1% acetic acid and purified water.

Staining procedure

Before dye application, the lesions were first subjected to 1% acetic acid application using cotton gauze for 20 seconds, in order to remove food debris and excess saliva and to provide a consistent oral environment. The mucosa of the target area was then gently dried with gauze to avoid contamination with saliva. Thereafter the dye was directly applied on the lesion with help of cotton roll for 20 seconds, followed by reapplication of 1% acetic acid to remove the excess (Figure 1). 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 while wide, shallow or faint blue stains or no stains were marked as negative (–) reaction (Figure 2). The results of methylene blue dye staining were then photographed, and biopsy was performed subsequently in the suspected area, to compare the diagnostic capability of methylene blue. All these steps were performed by an Oral Medicine specialist.

Biopsy

Punch biopsy, using a 8 mm instrument, was performed by an Oral Medicine specialist in the most obvious staining area of the suspected lesion of the patient under local anaesthesia. If there was no dye uptake in the lesions, the biopsy specimen was taken from the area judged by the clinician's experience. The specimen was then fixed in 10% neutral buffered formalin and prepared in the oral pathology laboratory for histopathologic diagnosis (Figure 1).

Histological examination

All the specimens were microscopically evaluated by an Oral Pathologist, who was blind to the results of methylene blue stain and reported as epithelial hyperplasia and hyperkeratosis, mild dysplasia, moderate dysplasia, severe dysplasia or carcinoma in situ for the leukoplakic and erythroplakic lesions, on the basis of Oral Epithelial Dysplasia classification system given by WHO

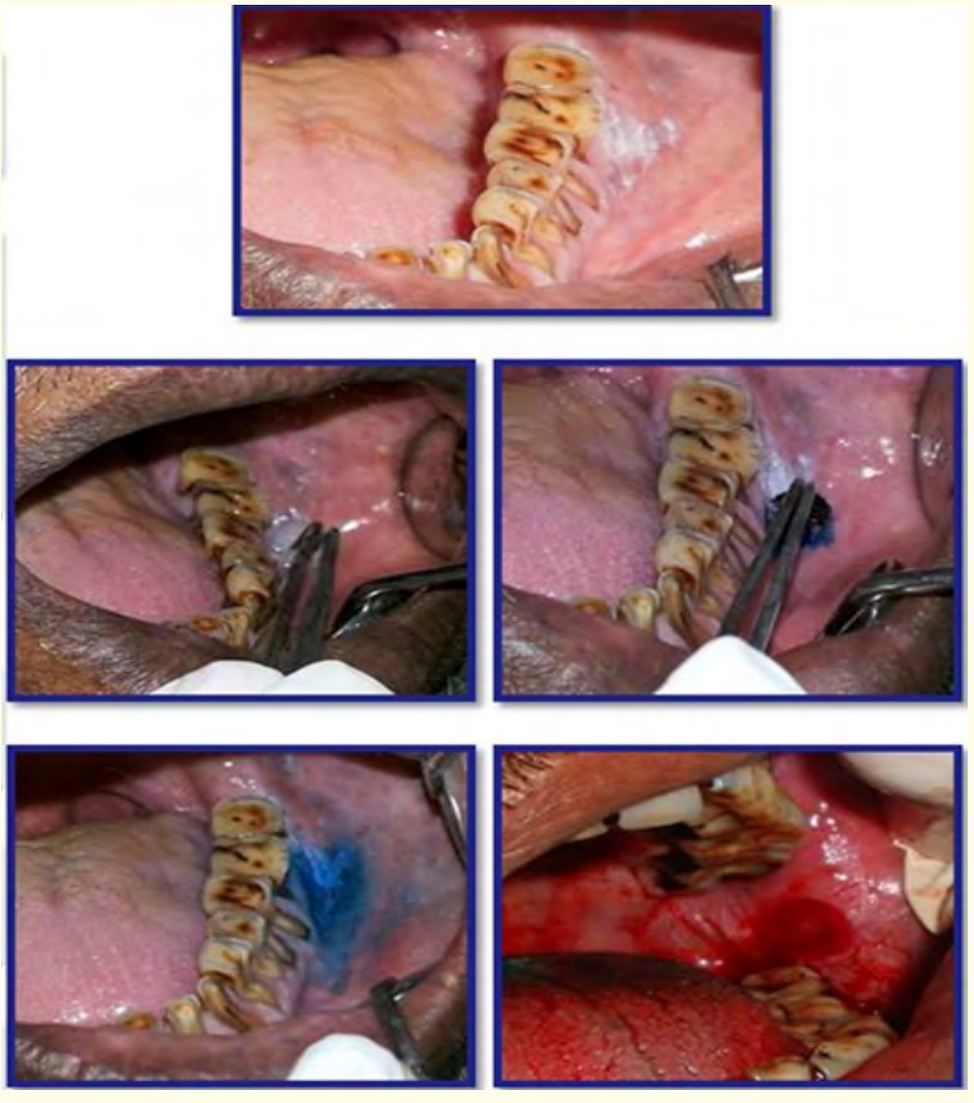

Figure 1: Method of dye application in clinically diagnosed potentially malignant oral disorder followed by biopsy in the area of maximum dye retention.

[9], and as oral lichen planus (indicating absence of dysplasia) or lichenoid dysplasia for the oral lichen planus tissue specimens.

Statistical Analysis

Statistical analysis included assessment of the sensitivity, specificity, positive and negative predictive values and the diagnostic accuracy of the staining method [10].

Inter-rater reliability analysis using the Kappa Statistic was performed to determine consistency among the vital staining and histopathological analysis [11]. Also, the p (probability) value was determined for the same. Interpretation of Kappa/Kappa Statistic Strength of Inter-Rater Agreement (Landis and Koch-Kappa's Benchmark Scale): [12]

- < 0.0 = Poor
- to 0.20 = Slight
- 0.21 to 0.40 = Fair
- 0.41 to 0.60 = Moderate
- 0.61 to 0.80 = Substantial
- 0.81 to 1.00 = Almost Perfect

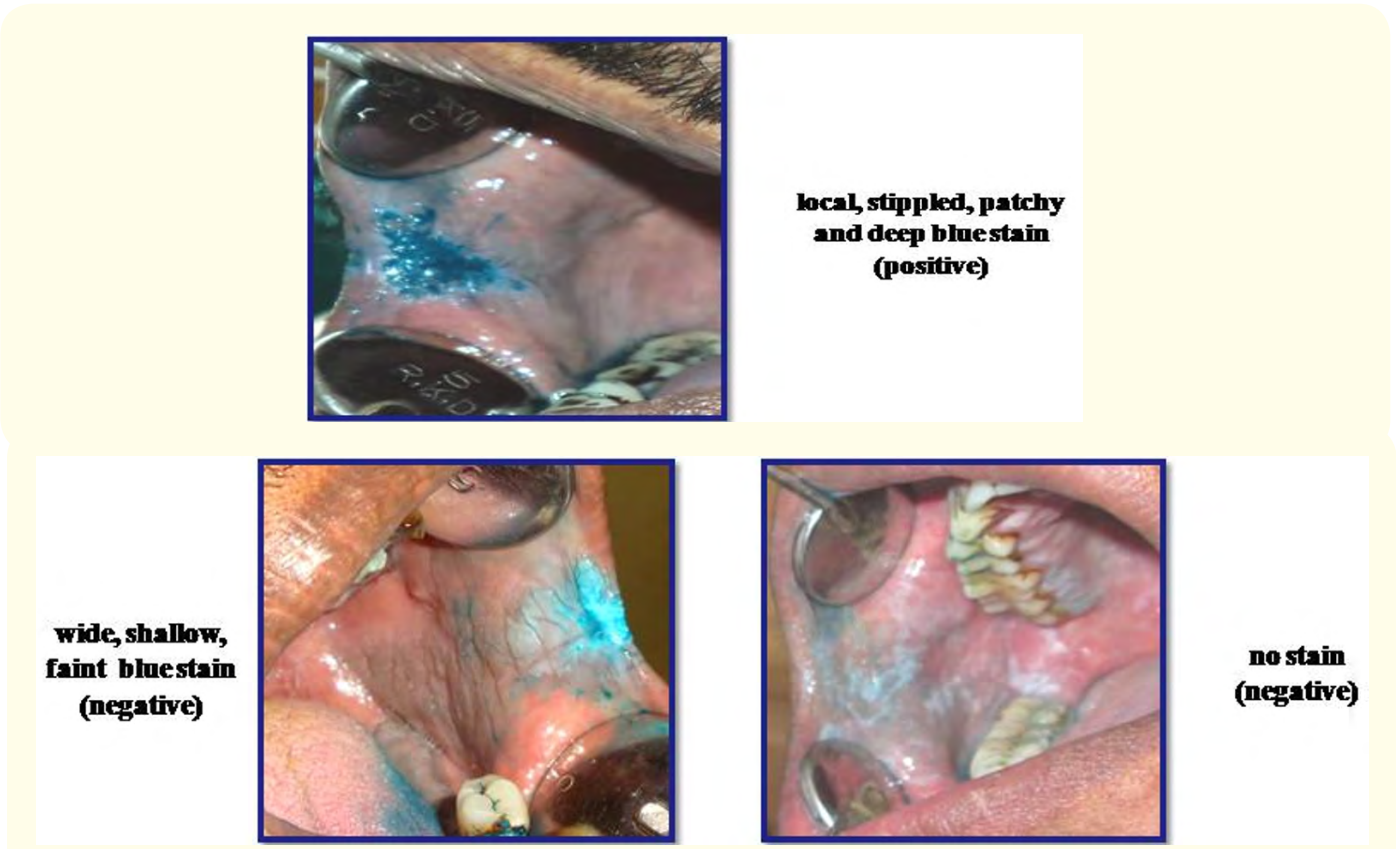

Figure 2: Patterns of dye retention.

Results

Subject Characteristics

50 patients (49 males, 1 female) were enrolled in this study who ranged between 15 to 74 years. The habit history comprised of the smoked and smokeless forms of tobacco, alone or in combination, with or without alcohol consumption. With regard to the clinical types of the PMDs diagnosed among the patients of our study group, 44% cases comprised of Homogeneous Leukoplakia, 38% cases comprised of Non Homogeneous Leukoplakia, 14% cases comprised of Oral Lichen Planus and 4% cases comprised of Erythroplakia, majority of them being distributed over the buccal mucosa (92%) followed by the tongue (2%).

Methylene blue staining related to grade of pathology

Out of the total 50 subjects, 33 cases i.e. 66% stained positive with the Methylene Blue dye while 17 cases i.e. 34% showed negative staining with the same (Table 1). According to the histopathological analysis of the lesions carried out thereafter, 38% lesions showed moderate dysplasia, 24% showed mild dysplasia, 14% showed severe dysplasia, 2% showed carcinoma in situ and 6% showed lichenoid dysplasia while 10% and 6% cases showed merely epithelial hyperplasia and hyperkeratosis and features of oral lichen planus (minus dysplasia) respectively.

Result of vital staining	Histopathological atypia		total (n)
	Present (n)	Absent (n)	
Positive(n)	30	3	33
Negative (n)	12	5	17
Total (n)	42	8	50

Table 1: Cross Tabulation of the Result of Vital Staining and Histopathological Atypia.

The following statistical terms were used to describe and analyze the relationship between the grade of pathology and the uptake of methylene blue staining.

From the total 50 subjects, 30/42 pathologically proven oral PMDs showed positive staining with localised and deep blue stain (Figure 3). Thus, the overall Sensitivity was 71.4% (Table 1).

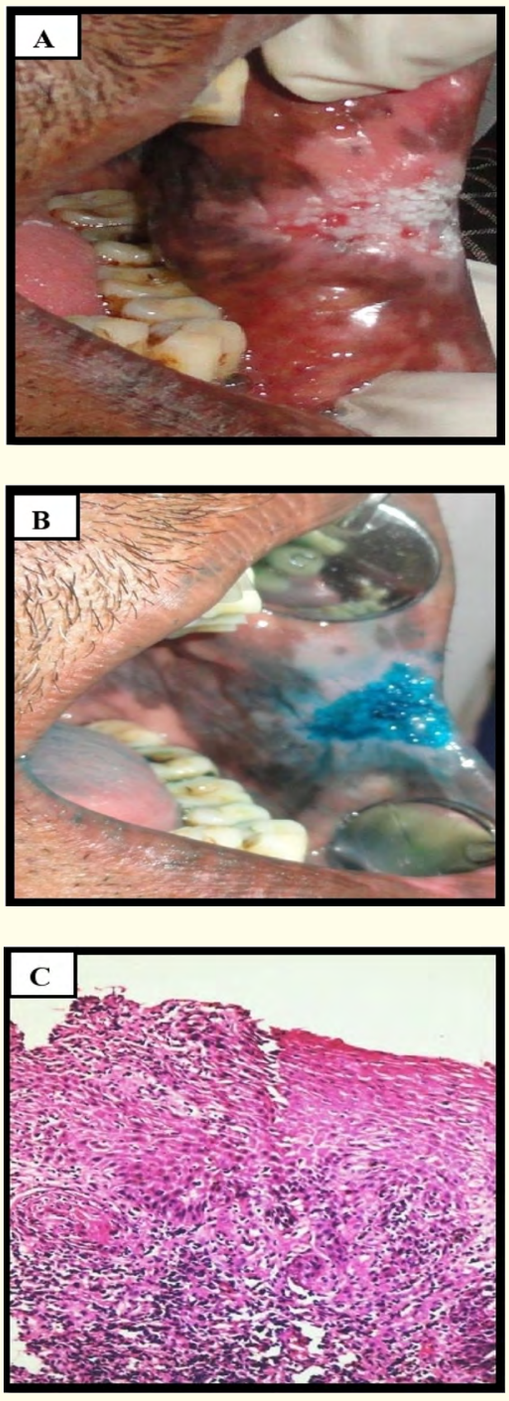

Figure 3: Presentation of a true-positive staining on a red and white homogenous patch on the left buccal mucosa:

(A) The lesion presented clinically as a red and white homogenous patch.

(B) Vital staining with methylene blue showed deep and focal staining of the lesion.

(C) The final pathology revealed a severe dysplasia. (Haemotoxylin and Eosin stain, original magnification x10).

In our study, 5/8 cases with no dysplasia showed negative staining; thus, the Specificity was 62.5% (Table 1).

Out of the 33 lesions which showed positive retention of the dye clinically, 30 showed atypia on histopathological analysis (True Positive) while 3 showed no histopathological atypia (False Positive). Thus, the Positive Predictive Value (PPV) was reported as 90.9% (Table 1).

Among the 17 lesions which were interpreted as showing negative staining by the Methylene Blue dye, 5 did not show any histopathological atypia either (True Negative), whereas 12 did (False Negative). Hence, the Negative Predictive Value (NPV) was reported as 29.4% (Table 1).

The overall diagnostic accuracy of the Methylene Blue dye for early diagnosis of PMDs was found to be 70% according to our study (Table 1).

The inter-rater reliability, Kappa, for the raters (vital staining and histopathological analysis) was found to be Fair (0.23) and the p-value (probability value) showed equivocal significance (0.063).

Discussion

Very few studies to assess the efficacy of Methylene Blue, a promising dye for the early diagnosis of oral PMDs, have been conducted and/or published till now. To the best of our knowledge, only three such studies have been published so far i.e. by Chen YW., *et al*. 2007, Riaz., *et al*. 2013 and Lejoy A., *et al*. 2016 [1,4,13]. Thus, the present study was performed with the aim of re-establishing Methylene Blue as an effective alternative diagnostic aid for early diagnosis of potentially malignant oral disorders in suspected cases and in turn, preventing full-fledged cancer formation.

The present study comprised of 50 subjects, clinically diagnosed with PMDs. In the studies done by Chen YW., *et al*. Riaz A., *et al*. and Lejoy., *et al*. the numbers of subjects with oral PMDs, were 52, 50 and 75 respectively [1,4,13].

In our study, no specific age range was described in the inclusion criteria. However, the age of the subjects ranged between 15 to 74 years, out of which the majority i.e. 40 % belonged to the age group of 35 to 44 years and the least i.e. 4% were found in the age group of 65 to 74 years. The age range of subjects in the study done by Riaz A., *et a*l. and Lejoy., *et al*. was close to our patient age range i.e. between 15 to 80 years, and 21 to 73 years, respectively [1,13]. However, Chen YW., *et al*. included individuals aged between 31 to 82 years in their study [4].

With regard to the gender of the subjects of the study group, the majority comprised of 98% males as compared to 2% females. A similar trend was also noted in the studies done by Chen YW., *et al*. in which the M: F was 51:7, in the study by Riaz A., *et al*. in which the M: Fa was 5:1 and that by Lejoy., *et al*. wherein it was 14:1 [1,4,13].

The tobacco habit history varied for the individuals and comprised of the smoked and smokeless forms, alone or in combination, with or without alcohol consumption. However, the majority patients had a history of consuming combination of smoked and smokeless products. Higher prevalence of the smoking habit compared to betel nut chewing among the subjects was observed by Chen YW., *et al*. in their study [4]. No specifics regarding the prevalence of the tobacco habit sub-types have been mentioned in the studies by Riaz., *et al*. and Lejoy., *et al*. [1,13].

In terms of the clinical types and sub-types of the PMDs diagnosed among the patients of our study group, 44%, 38% and 4% cases comprised of Homogeneous Leukoplakia, Non-homogeneous Leukoplakia and Erythroplakia respectively, as against 36%, 21% and 10% respectively of the same in the study by Chen YW., *et al*. In our study, 14% cases also comprised of Oral Lichen Planus whereas no cases of the same were included in the study by Chen YW., *et al*. [4]. The study done by Riaz A., *et al*. included only two types of PMDs i.e. 88% Leukoplakia and 4% Smoker's Palate whereas Smoker's Palate lesions were not included in our study group [1]. In the study by Lejoy., *et al*. Homogenous Leukoplakia in 52% patients, Non-homogenous Leukoplakia in 36% patients and Erythroplakia in 6.7% patients was seen [13].

With regard to the intraoral sites involved by the PMDs, the buccal mucosa was most commonly involved followed by the tongue. This pattern of site distribution was similar to that observed in the studies done by Chen YW., *et al*. and Riaz A., *et al*. in which also the buccal mucosa was the most common site of involvement by the PMDs. The tongue was the second common site of involvement as well in the study done by Chen YW., *et al*. while it was the third commonly involved site in the study by Riaz A., *et al*. The other sites involved by the PMDs in these two studies were the gingivae, lips, palate, floor of the mouth and retromolar trigone, which were not seen to be involved in our sample group [1,4].

Among the 50 subjects, 30 out of 42 pathologically proven oral PMDs showed positive staining with localised and deep blue stain. Thus, the overall Sensitivity was 71.4% (Table 1) while the sensitivity of 90%, 91.3% and 92% was reported by Chen YW., *et al*. Riaz A., *et al*. and Lejoy., *et al*. respectively [1,4,13].

In our study, 5 out of 8 cases with no dysplasia showed negative staining; thus, the Specificity was 62.5 % (Table 1) which was close to the specificity of 69% reported by Chen YW., *et al*. however, Riaz A., *et al.* and Lejoy., *et al*. reported a relatively higher specificity of 75% and 70% respectively in their studies [1,4,13]. Out of the 33 lesions which showed positive clinical retention of the dye, 30 showed atypia on histopathological analysis (True Positive) while 3 showed no histopathological atypia (False Positive). Thus, the Positive Predictive Value (PPV) was reported as 90.9% (Table 1), close to that reported by Lejoy., *et al*. (91%) and Riaz A., *et al*. (97.6%) [1,13]. Chen YW., *et al.* reported a lower PPV of 74% [4]. The false positives could be related to the retention of the stain in inflamed and trauma areas. Other factors could be irregular, papillary or digital surfaces of the lesions, which may cause the mechanical retention of dye, contamination of saliva and plaque, retention of dye material in papilla of the tongue or minor salivary gland ducts over the mucosa [1].

Among the 17 lesions which displayed negative staining by the Methylene Blue dye, 5 lesions did not show any histopathological atypia either (thus True Negative), whereas 12 lesions did (False Negative). Hence, the Negative Predictive Value (NPV) was reported as 29.4% (Table 1), as against 42.8%, 87% and 80% reported by Riaz A., *et al.* Chen YW., *et al.* and Lejoy., *et al*. respectively [1,4,13]. The false negative cases could have resulted due to ambiguous blue faint/shallow stains, which may have been misinterpreted as negative but were clinically suspicious of premalignancy, as was proved histopathologically post-biopsy subsequently [1].

The overall diagnostic accuracy of the Methylene Blue dye for early diagnosis of PMDs was found to be around 70% according to our study (Table 1), while it was shown to be 90% as per the study done by Riaz A., *et al.* [1].

In the present study, the association of Methylene Blue uptake and histopathologic diagnosis among the PMDs was analysed using the Kappa Statistics test, while the Fisher's Exact test was utilised for statistical analysis of the data by both Chen YW., *et al*. and Riaz A., *et al*. [1,4]. Thus, the resulting inter-rater reliability, Kappa, in our study, for the raters (vital staining and histopathological analysis) was found to be Fair (0.23) and the p-value (probability value) showed equivocal significance (0.063), while those derived in the studies by Riaz A., *et al*. and Chen YW., *et al*. were statistically significant (< 0.001) and very significant (< 0.0001) respectively [1,4]. The p value as derived using the Yate's corrected chi square test in the study by Lejoy., *et al.* was significant ($p < 0.01$) [13].

The above findings of our study and also of the other three studies, show that Methylene Blue dye can be considered as an aid for screening of oral PMDs and as a substitute for the TB dye. The TB dye is proposed to be relatively toxic to fibroblasts with a probability of inducing mutagenesis especially if the vitally stained cells are exposed to high energy irradiation like light, and also relatively expensive [4,14].

Conclusion

We conclude that the MB dye is an efficacious diagnostic adjunct and a suitable alternative for the routinely used TB dye, for large-scale, community-based OPMD and oral cancer screening programs among high-risk individuals.

Acknowledgements

We would like to acknowledge and thank the Department of Chemistry, Isabella Thoborn College, Lucknow (India) for providing their laboratory facility for the preparation of the dye.

Conflict of Interest

The authors do not wish to declare any conflicts of interest.

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Volume 2 Issue 11 November 2018