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ORIGINAL ARTICLE

Utility of toluidine blue as a diagnostic adjunct in the detection of potentially malignant disorders of the oral cavity – a clinical and histological assessment

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BACKGROUND: The value of chairside adjunctive tests in the detection of oral potentially malignant disorders (OPMDs) remains uncertain.

OBJECTIVES: To determine the effectiveness of toluidine blue in detecting leukoplakia and erythroplakia and its accuracy in identifying cases with oral epithelial dysplasia.

MATERIALS AND METHODS: Ninety-two patients attending two oral medicine clinics in London, presenting with white and red patches of the oral mucosa, were investigated by the application of toluidine blue. Eighty-two patients were clinically diagnosed as OPMDs and 10 were frictional keratoses. A surgical biopsy was performed to assess epithelial dysplasia.

RESULTS: Of 64 oral leukoplakias, 34 (53.1%) were positive for toluidine blue and among nine erythroplakias seven stained positive. Of 41 oral dysplasia cases, a little more than half of the lesions (*n* = 23) were stain positive, an estimated sensitivity of 56.1%. TBlue test had a higher sensitivity for detecting higher-grade dysplastic lesions (5/8 moderate dysplasia, sensitivity 62.5%; 5/7 severe dysplasia; sensitivity 71.4%) compared with lower grades of dysplasia, but the differences were not significant (*P* = 0.60).

CONCLUSIONS: We report here the utility of TBlue for the detection of oral leukoplakia and erythroplakia. The test has the potential to detect OPMDs and yielded a sensitivity of 56.1% and specificity of 56.9% to detect oral epithelial dysplasia.

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Introduction

Potentially malignant disorders of the oral cavity (OPMDs) that precede the development of oral squamous cell carcinomas (OSCC) are well characterized (Warnakulasuriya *et al*, 2007). Of these, oral leukoplakia and erythroplakia have received considerable attention particularly in relation to their management (Van der Waal, 2010). OPMDs may be asymptomatic or display a benign clinical appearance making it difficult sometimes for the clinician to differentiate them from common reactive or inflammatory (benign) disorders of the oral mucosa. Their early detection may help to identify high-risk subjects thus allowing the clinician to initiate appropriate intervention measures to reduce the risk of later malignancy (Warnakulasuriya, 2011). Several adjunctive diagnostic clinical aids and agents are now available to facilitate both the visualization of oral cancers and the detection of OPMDs (Kahn and Epstein, 2007). Among these, the toluidine blue test is the most comprehensively studied method (Leston and Diz Dios, 2010).

Toluidine blue (TBlue) is a catoionic metachromatic dye that selectively binds *in vivo* to acidic tissue components (sulphate, carboxylate and phosphateradicals) of DNA and RNA and also may be retained in intracellular spaces of the dysplastic epithelium (Mashberg,1983; Martin *et al*, 1998; Patton, 2003; Missmann *et al*, 2006). TBlue dye also binds preferentially to tissues undergoing rapid cell division (such as inflammatory, regenerative and neoplastic tissues) (Myers, 1970; Mashberg and Samit,1995; Gandolfo *et al*, 2006; Patton *et al*, 2008) and particularly to sites with DNA damage demonstrating loss of heterozygosity or allelic

losses in OPMDs and OSCC (Epstein *et al*, 1997). The binding results in the staining of abnormal tissue with a blue colouration that contrasts with adjacent normal mucosa. Its use *in vivo* is based on the fact that dysplastic cells contain quantitatively more nucleic acids than normal tissues (Epstein *et al*, 1997).

The aims of this study were to evaluate the accuracy of TBlue staining as an adjunctive test in its ability to identify white and red mucosal lesions already visually diagnosed by a specialist as leukoplakia, erythroplakia or erythroleukoplakia, and to estimate the sensitivity and specificity of the technique, particularly with respect to the presence or absence of oral epithelial dysplasia.

Materials and methods

Consecutive patients aged over 16 years presenting in oral medicine clinics at two London Hospitals with white, mixed red and white and red patches of the oral mucosa were invited to participate in the study. Ninety-two who consented to the study were investigated by a standard protocol that involved clinical visual examination and TBlue staining, and repeat visual examination and photography followed by biopsy. The study was approved by Institutional Research and Ethics Committee (08/H0808/20).

Following a comprehensive clinical examination under an incandescent light source, the clinical diagnosis was established by the operator (KHA) and validated by a second experienced examiner (SW). The principal site of morphologically altered mucosa was selected (by consensus of both examiners) and photographed. All further investigations were performed on this clinically detected area of mucosal abnormality. Among the 92 patients, 10 were diagnosed with frictional keratoses and the rest ($n = 82$) were classified as OPMDs and clinically characterized to oral leukoplakia, erythroplakia or erythroleukoplakia (Warnakulasuriya *et al*, 2007). TBlue staining test was performed using the TBlue® oral lesion marking system manufactured by Zila Inc., (Phoenix, AZ, USA). The TBlue kit consisted of three swab tubes: Swab tube1, 1% acetic acid solution (prerinse swab), Swab tube 2, 0.5% tolonium chloride solution and Swab tube 3, 1% acetic acid solution (postrinse swab). The staining procedure was carried out according to the manufacturer's instructions.

A surgical biopsy was performed for histopathological assessment, and the selection of the biopsy site took into consideration any area within the lesion that stained positively with TBlue. Any blue-stained area, if present, was included in the biopsy sample. The presence or absence of dysplasia (WHO, 2005) in the biopsy specimen was confirmed by two experienced oral pathologists as is the routine practice during microscopic assessment for all OPMDs received in our diagnostic laboratory. Prior to data entry, the dysplasia grades given by the two pathologists during the initial biopsy diagnosis were further reviewed by one of the pathologists (PRM) blinded to the test result.

Data collected were entered through the IBM SPSS 18 (IBM Corporation 1 New Orchard Road, Armonk, NY, USA) (Statistical Package for the Social Sciences). Sensitivity and specificity of the toluidine blue test results, compared to clinical diagnosis by a specialist and dysplasia grade from biopsy, were calculated. For calculation of specificity, the group with frictional keratoses was compared against OPMDs. Differences and associations between the TBlue test and dysplasia grade were examined using Fisher's exact test with significance set at $P < 0.05$. All tests were two-sided.

Results

The profile of 82 patients with white, mixed red and white and red patches enrolled in this study is given in Table 1. Of these, 64 were clinically diagnosed as leukoplakia, nine as erythroplakia and another nine as erythroleukoplakia. All the nine cases of erythroleukoplakia had candida diagnosed in the biopsy. Ten lesions were clinically diagnosed as frictional keratoses as the white patch was along the occlusal plane and a source of friction was evident. All 92 cases underwent surgical biopsy from which oral epithelial dysplasia was confirmed in 41 patients.

TBlue examination was performed on all 92 patients (Table 2). Among the frictional keratoses group (included as controls to assess the specificity of the test), only one of them stained positive. Among the test group ($n = 82$), 46 (56.1%) were positive for TBlue as they retained the dye. An almost equal number did not retain TBlue and therefore were recorded as negative for

Table 1 Patient characteristics

	White/red lesions[a]		*Dysplasia*[b]	
	n = *82*	*%*	n = *41*	*%*
Gender				
Male	50	61.0	26	63.4
Female	32	39.0	15	36.6
Ethnicity				
White	55	67.1	28	68.3
Non-white[c]	27	32.9	13	31.7
Tobacco history				
Current smokers	43	52.4	22	53.7
Ex-smokers	18	22.0	13	31.7
Never smoked	21	25.6	6	14.6
Alcohol history				
Current users	65	79.3	32	78.0
Ex-users	5	6.1	4	9.8
Never used	12	14.6	5	12.2
Lesion site				
Buccal mucosa	30	36.6	11	26.8
Tongue	27	32.9	16	39.0
Floor of mouth	9	11.0	8	19.5
Palate	9	11.0	4	9.8
Alveolar ridge	7	8.5	2	4.9
Lesion classification[a]				
Leukoplakia	64	78.0	34	82.9
Erythroplakia	9	11.0	6	14.6
Eythroleukoplakia[d]	9	11.0	1	2.4

[a]Test group (excluding frictional keratosis group).
[b]Mild – 26; moderate – 8; severe – 7.
[c]Non-white included 14 Afro-Caribbean, 11 Asians and two of mixed ethnicity.
[d]All erythroleukoplakia cases were candida positive in biopsy.

Table 2 Results of TBlue staining

	Test group[a]		*FK group*[b]		*Dysplasia*	
	n = *82*	*%*	n = *10*	*%*	n = *41*	*%*
Test result						
Stain +ve	46	56.1	1	10	23	56.1
No stain	36	43.9	9	90	18	43.9
Intensity of TBlue stain						
Dark blue	26	56.5	1	100	14	60.9
Light blue	20	43.5	0	0	9	39.1
Size of TBlue-positive lesions						
Complete	14	30.4	0	0	6	26.1
Partial	24	52.2	1	100	14	60.9
Beyond	8	17.4	0	0	3	13.0
Margins of TBlue-positive lesions						
Defined + irregular	8	17.4	0	0	4	17.4
Defined + circular/oval	32	69.6	1	100	16	69.6
Diffused	6	13.0	0	0	3	13.0

[a]Test group included 64 leukoplakias, nine erythroplakias and nine leukoerythroplakias.
[b]FK group included frictional keratoses without dysplasia.

the test. Among the 46 TBlue-positive lesions, 26 appeared dark blue after removal of excess dye, whereas 20 oral lesions appeared light blue. In 40 of 46 positively stained lesions, the margins were well defined. Some examples of cases stained with TBlue with positive or negative results are illustrated in Figure 1.

Of 64, 34 oral leukoplakias (53.1%) stained positive (with almost equal number indicating dark or light staining) while seven of nine erythroplakias stained positive (77.8%). Among the erythroleukoplakia group ($n = 9$), only five stained positive for TBlue. No significant differences were noted in the ability of TBlue detecting red lesions compared with white lesions (Fisher's Exact Test $P = 0.29$). However, the sensitivity (se) of the test was noted to be better for detecting erythroplakia (77.8%) compared with leukoplakia (53.1%). The specificity (sp) compared with frictional keratoses group was similar for both groups at 90.0%.

Dysplasia group

Of 41 oral dysplasia cases, a little more than half of the lesions ($n = 23$) were stain positive giving a sensitivity (se) of 56.1% to the test. However, it was noted that a higher proportion was positive among the higher dysplasia grades (5/8 moderate dysplasia, se 62.5%; 5/7 severe dysplasia, se 71.4%) compared with lower grades (13/26 mild dysplasia, se 50%). In the no dysplasia group of oral lesions, TBlue staining was

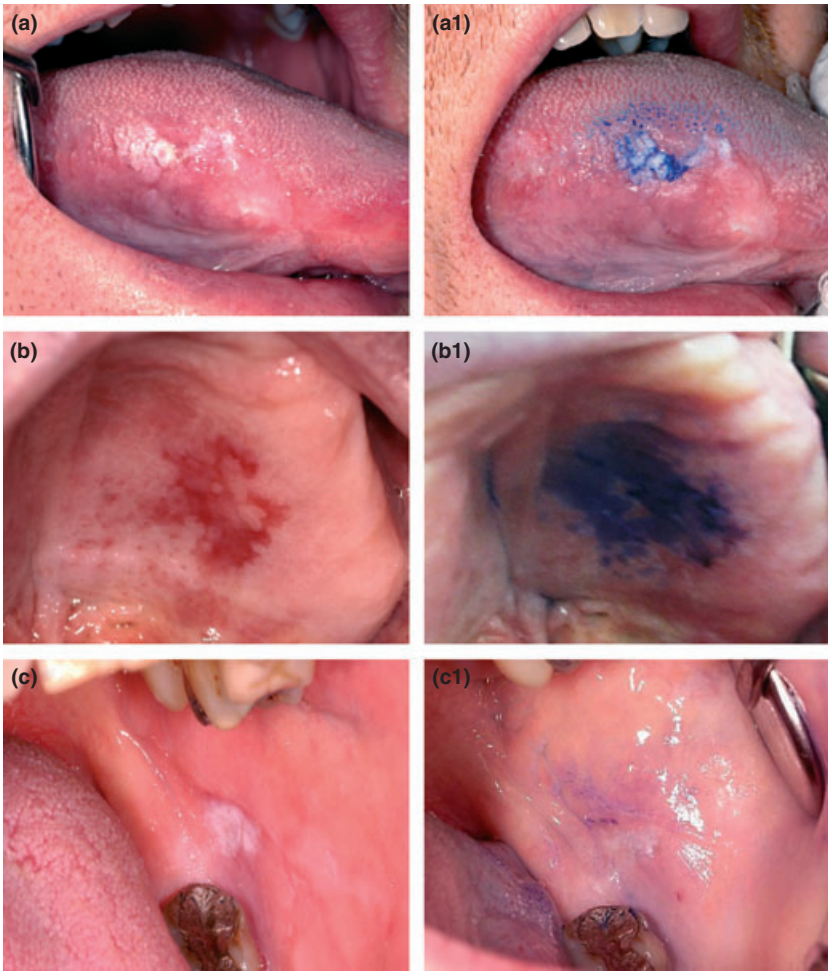

Figure 1 Test results showing variations observed following TBlue staining. Figures **a**+**a1** and **b**+**b1** show staining and retention of TBlue dye for the white and red patches, respectively. Staining of the white patch was partial where as the red patch was completely stained. Figures **c**+**c1** show lack of staining or retention of TBlue dye for the white patch observed under incandescent light

positive in 22 (43.1%) of the 51 lesions, whereas remaining ($n = 29$) did not retain any stain. No significant differences were noted between the dysplasia and non-dysplasia groups in relation to TBlue test results ($P = 0.29$). There were also no significant differences on staining characteristics among different grades ($P = 0.60$).

Specificity (sp) – the proportion of non-dysplastic lesions which are correctly identified as negatively stained by TBlue – was 56.9% (Table 3).

Discussion

TBlue is a vital dye that stains nucleic acids and abnormal tissues. Staining with TBlue has been used for more than four decades as an adjunctive test for the detection of OPMDs and/or oral cancers. Previously the diagnostic system was available in the form of an oral rinse (OraScan®; Zila Inc.). In an earlier field study, our group had tested the utility of OraScan in detecting oral epithelial dysplasia (Warnakulasuriya and Johnson, 1996). However, recently it has been marketed in the form of swab along with ViziLite as 'ViziLite Plus' – an oral lesion identification and marking system (Zila® Inc). We found the use of TBlue swabs more convenient for use by the clinicians and user-friendly for our patients.

In our study, more than half of the leukoplakias and 78% of erythroplakias showed a positive test result for TBlue staining giving an over all sensitivity and specificity for the total tested group of 56% and 90% respectively. Sensitivity of TBlue in detecting oral mucosa lesions in published studies range from 38 to 98 per cent (median, 85 per cent) and the specificity from 9 to 93 per cent (median, 67 per cent), respectively (Patton *et al*, 2008). The wide ranges noted in these data could be interpreted as a consequence of potential differences in the inclusion criteria applied to sampling of patients, the variability in the methods employed and the array of clinical expertise among the reporting authors, leading to significant variations in the way test results are interpreted. It is clear that case mixes of study populations are very different in terms of clinical conditions included in the samples with a mix of OPMDs. For these reasons, we only included white and red lesions in our analysis reported here. Most studies have shown a higher sensitivity for detection of oral cancers (Portugal *et al*, 1996; Warnakulasuriya and Johnson, 1996), and therefore inclusion of higher numbers of carcinomas in the study will result in a higher sensitivity value. On the other hand, if a large number of benign keratoses and dysplasias are included, the sensitivity will fall if some dysplasias fail to retain the dye (Cancela-Rodriguez *et al*, 2011). In addition, the inclusion of benign lesions will also result in low specificity as some of these lesions may retain the dye. In our study, we included a range of white and red lesions and excluded any cancers by histology, and the sensitivity and specificity were lower than reported in previous studies that were limited to cancers or high-risk lesions.

The sensitivity of TBlue in detecting the dysplasia cases was relatively low at 56.1%. A previous study by us (Warnakulasuriya and Johnson, 1996) had reported test positive results for 29 of 39 dysplasias confirmed by biopsy as having a sensitivity of 74.4%. However, we noticed that when dysplasia lesions were grouped (Warnakulasuriya *et al,* 2008) into high-risk (moderate/severe dysplasia) and low-risk (mild dysplasia), the sensitivity of TBlue increased from a low 50% for mild to a high 71% for higher grades. A similar observation has been commented on in a previous study (Epstein and Güneri, 2009) in which the authors reported a higher number of TBlue-positive cases among the moderate and severe dysplasia compared with mild dysplasia cases. Other studies have also shown TB staining to be a sensitive adjunct for identifying high-risk lesions compared to its ability to detect low-risk lesions (Onofre *et al*, 2001; Epstein *et al*, 2003).

Some modifications to the technique have been proposed in previous studies to improve the false-positive results. One way of reducing false positives, as suggested by Mashberg (1980), is to wait 10–14 days after initial screening with TBlue to avoid false-positive test results

Table 3 TBlue staining in relation to white/red lesions and dysplasia

Diagnosis	*Cases* (n)	*Stained-positive*		*Stained-negative*	*Se*	*Sp*
		Dark blue	*Light blue*			
Clinical groups[a]						
Leukoplakia	64	18	16	30	53.1	90.0
Erythroleukoplakia	9	3	2	4	55.6	90.0
Erythroplakia	9	5	2	2	77.8	90.0
All OPMDs	82	26	20	36	56.1	90.0
Frictional keratosis	10	1	0	9		
Dysplasia[b]	41	14	9	18	56.1	56.9
Mild dysplasia	26	6	7	13		
Moderate dysplasia	8	3	2	3		
Severe dysplasia	7	5	0	2		
No dysplasia	51	12	10	29		

se – sensitivity, sp – specificity.
[a]Based on Warnakulasuriya *et al* (2007), criteria confirmed by a specialist.
[b]Based on World Health Organization (2005) criteria.

among inflammatory or traumatic oral lesions (Mashberg, 1980). We did not employ this 2-week wait as this hospital series needed confirmation of diagnosis by biopsy without any delay. This may help to explain high false-positive results in our study.

There has been some debate about the intensity of the TBlue-positive lesions in interpreting test results as true positives. Gandolfo *et al* (2006) in their *in vivo* study recommended dark royal blue-stained lesions to be considered as true positives. The authors reported that although all of the malignant lesions stained dark royal blue, there were also four benign lesions that stained the same. Therefore, a further larger study is needed to confirm their recommendations. In our study, we interpreted both dark blue and light blue as true positives. Furthermore, a re-analysis of our data by considering staining intensity indicates a random distribution of the cases when classified by dark or light staining.

The problematic issues associated with studies of TBlue are listed in Table 4.

A review of the studies utilizing TBlue has concluded the test to be a good adjunctive test (Epstein and Güneri, 2009). However, other authors have reported reservations about its value as a diagnostic tool. Lingen *et al* (2008) in a review could not agree that any adjunctive test was superior to clinical visual examination performed by a trained clinician. In our study, we too found the sensitivity of TBlue as a diagnostic test to be low when used for the full range of white and red lesions. However, in evaluating the dysplasia only group, TBlue appears to be powerful in determining the higher risk group blindly graded by two pathologists, although these differences did not reach statistical significance. Combination of two adjunctive tests such as toluidine blue and brush biopsy has been found to give a good concordance for suspicious oral mucosal lesions (Güneri *et al*, 2011). Two population-based studies reported that the use of TBlue may increase the case detection of OPMDs and cancer compared with visual examination (Vacher *et al*, 1999; Su *et al*, 2010). Based on our results, it is not clear whether the widespread use of TBlue as an adjunct in dental practice would result in an increased number of case detection, but in the hands of specialists, its use may reduce a number of unnecessary biopsies and the test result decreases the uncertainty for further investigation.

Table 4 Problems with studies of TBlue

No studies carried out in primary care setting
No randomized control trials
Variability in study methodology (some use single rinse while others use double rinse method)
Some studies include only carcinomas or dysplasias while some include both
Studies that include both cancers/OPMDs did not report sensitivity and specificity separately
Two weeks waiting period for a double rinse (although improving specificity of the test) may add to delays in diagnosis
Histological diagnosis is not always used as a gold standard
Confusion over interpretation of positive results (dark blue and light blue)

Conclusion

Based on our results, TBlue is a useful adjunct to clinical visual examination by aiding in the visualization of lesions. Our study yielded a sensitivity of 56.1% and specificity of 56.9% to detect oral epithelial dysplasia. However, it is not clear whether wide spread use of TBlue as an adjunct in dental practice would result in better diagnosis of OPMDs. In a specialist practice, based on our data, the test has the potential to detect OPMDs but cannot accurately predict the presence of oral epithelial dysplasia. Further well-designed studies are needed to examine the role of TBlue as an oral examination system or for screening studies in primary care.

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Conflict of interest

This study did not receive any grant funding by the industry but the test kits were supplied free of charge by Zila® Inc.

Author contributions

KH Awan: study execution, data entry, drafting of the paper; YH Yang: setting up the data entry system, power calculation and data analysis; PR Morgan: pathology reporting, revising paper; S Warnakulasuriya: research design, clinical aspects of sample acquisition and assessments, study coordination, interpretation of data, revising paper, acting as PI.

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