Toluidine Blue 0.05% Vital Staining for the Diagnosis of Ocular Surface Squamous Neoplasia in Kenya

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IMPORTANCE Clinical features are unreliable for distinguishing ocular surface squamous neoplasia (OSSN) from benign conjunctival lesions.

OBJECTIVE To evaluate the adverse effects, accuracy, and interobserver variation of toluidine blue 0.05% vital staining in distinguishing OSSN, confirmed by histopathology, from other conjunctival lesions.

DESIGN, SETTING, AND PARTICIPANTS Cross-sectional study in Kenya from July 2012 through July 2014 of 419 adults with suspicious conjunctival lesions. Pregnant and breastfeeding women were excluded.

EXPOSURES Comprehensive ophthalmic slitlamp examination was conducted. Vital staining with toluidine blue 0.05% aqueous solution was performed before surgery. Initial safety testing was conducted on large tumors scheduled for exenteration looking for corneal toxicity on histology before testing smaller tumors. We asked about pain or discomfort after staining and evaluated the cornea at the slitlamp for epithelial defects. Lesions were photographed before and after staining.

MAIN OUTCOMES AND MEASURES Diagnosis was confirmed by histopathology. Six examiners assessed photographs from a subset of 100 consecutive participants for staining and made a diagnosis of OSSN vs non-OSSN. Staining was compared with histopathology to estimate sensitivity, specificity, and predictive values. Adverse effects were enumerated. Interobserver agreement was estimated using the κ statistic.

RESULTS A total of 143 of 419 participants (34%) had OSSN by histopathology. The median age of all participants was 37 years (interquartile range, 32-45 years) and 278 (66%) were female. A total of 322 of the 419 participants had positive staining while 2 of 419 were equivocal. There was no histological evidence of corneal toxicity. Mild discomfort was reported by 88 (21%) and mild superficial punctate keratopathy seen in 7 (1.7%). For detecting OSSN, toluidine blue had a sensitivity of 92% (95% CI, 87%-96%), specificity of 31% (95% CI, 25%-36%), positive predictive value of 41% (95% CI, 35%-46%), and negative predictive value of 88% (95% CI, 80%-94%). Interobserver agreement was substantial for staining (κ = 0.76) and moderate for diagnosis (κ = 0.40).

CONCLUSIONS AND RELEVANCE With the high sensitivity and low specificity for OSSN compared with histopathology among patients with conjunctival lesions, toluidine blue 0.05% vital staining is a good screening tool. However, it is not a good diagnostic tool owing to a high frequency of false-positives. The high negative predictive value suggests that a negative staining result indicates that OSSN is relatively unlikely.

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Ocular surface squamous neoplasia (OSSN) is an aggressive eye cancer, particularly affecting young adults in Africa, causing visual disability and high morbidity and mortality. The diagnosis is problematic. In most African countries, pathology services are limited; most clinicians depend on their clinical judgment.\(^{1,2}\) However, the appearance of OSSN overlaps with several benign conditions, making a clinical impression unreliable. Surgical excision is the mainstay of OSSN treatment. For example, a simple diagnostic test would help clinicians plan management by better delineating the boundaries of the lesion during excision. The test may also help in distinguishing early recurrent tumor from nonmalignant abnormal tissue, such as fibrosis, possibly avoiding the need for additional surgery.

Vital stains are used to color living tissues. Several dyes are used extensively in ophthalmic surgery.\(^{3,4}\) Toluidine blue (ToB) is an acidophilic metachromatic dye that stains abnormal tissue dark royal blue by penetrating into the nuclei of cancerous cells where it has a selective affinity for nucleic acids and by accumulating in the intercellular spaces.\(^{5}\) Malignant tissues stain more frequently than healthy epithelia because of their abundant nuclear material from increased mitoses and poor cell-to-cell adhesion.\(^{6,7}\) Mucin and inflammatory cells also take up ToB.\(^{5,7}\) Toluidine blue has been used safely for many years to aid the clinical diagnosis of oral and oropharyngeal cancer and to demarcate tumors during surgical excision.\(^{8,9}\)

A case report from Japan described the first use of topical ToB 0.05% vital staining for OSSN.\(^{10}\) The dye was reported to clearly demarcate the abnormal tissue, assisting the excision. The authors commented that ToB did not stain other conjunctival lesions, such as pterygium (no data presented), and it was not toxic to the ocular surface. Two relatively small studies have evaluated vital staining for OSSN using ToB 1% in Brazil and methylene blue 1% in South Africa.\(^{11,12}\) However, given the variation in clinical phenotype and prevalence of conjunctival lesions, it is necessary to test this in the local setting.

The aim of this study was to investigate the use of ToB 0.05% solution in detecting neoplastic tissue by evaluating its safety, accuracy, and interobserver variation.

**Methods**

**Ethical Approval**

This study was formally reviewed and approved by the Kenyatta National Hospital–University of Nairobi Ethics and Research Committee and the London School of Hygiene and Tropical Medicine Ethics Committee. This study adhered to the tenets of the Declaration of Helsinki. All participants gave informed written consent to take part in the study before enrollment and did not receive a stipend to participate.

**Participants**

The study was conducted between July 2012 and July 2014 in 4 eye care centers in different parts of Kenya: Kenyatta National Hospital in Nairobi, PCEA Kikuyu Eye Unit in Central Kenya, Kitale District Hospital in the north Rift Valley, and Sabatia Eye Hospital in western Kenya bordering Lake Victoria. It was part of a larger project on the epidemiology and management of OSSN in Kenya. These centers receive referral cases from the surrounding hospitals.

Consecutive adult patients (≥18 years of age) seen in these 4 eye clinics with conjunctival lesions (first presentation or recurrence) suspected to be OSSN scheduled for surgery who gave consent to participate in the study were included. Pregnant women and breastfeeding mothers were excluded.

**Toluidine Blue Eyedrops**

Toluidine blue 0.05% aqueous solution was prepared in the Kikuyu Eye Unit eyedrop production facility. Toluidine powder (Sigma Aldrich) 0.05 g was diluted in 100 mL of freshly distilled water and aliquoted into 5-mL eyedrop bottles. The bottles were sterilized in a water bath at 98°C for 30 minutes and checked for particles. Any bottles with particles were discarded. A bottle was used for up to 28 days once opened. New batches were prepared every 6 months.

**Clinical Assessment**

A comprehensive ophthalmic examination was conducted using a slitlamp. Clinical features of lesions were assessed including inflammation, leukoplakia, and involvement of adjacent structures. Vital staining with ToB 0.05% solution was performed at the slitlamp before surgery. One drop of the dye was applied to the ocular surface, waiting 30 seconds before wiping off the excess spillover from the eyelids with a soft tissue. Topical anesthetic was not applied before staining to evaluate whether ToB was painful. Staining with fluorescein was not done to avoid interference with the ToB dye.

**Surgery and Histopathology**

All lesions were excised under infiltration local anesthetic using an operating microscope with a 3-mm clear margin. The defect was reconstructed by primary closure. Cryotherapy was not applied as the participants with OSSN were invited to enroll in an additional treatment trial postoperatively. Specimens were placed directly into buffered formalin and subsequently examined at the histopathology laboratory at the MP Shah Hospital, Nairobi. One pathologist examined all the histology slides. Participants with mild, moderate, or severe conjunctival intraepithelial neoplasia (CIN 1, 2, or 3, respectively), carcinoma in situ, or invasive squamous cell carcinoma (well, moderately, and poorly differentiated) were classified
as having OSSN. The diagnosis of actinic keratosis was based on the presence of elastic stromal degeneration, acanthosis, hyperkeratosis, and parakeratosis in the presence of normal cellular polarity. By the accepted criteria for dysplasia, such lesions were classified as CIN only if there was loss of polarity.

Safety Study
There is extensive experience on the safety of using ToB in the oral cavity but only relatively limited data on the eye.9,11-14 Therefore, we conducted initial testing on large tumors scheduled for exenteration. The exenteration specimens were examined by a histopathologist for evidence of corneal toxicity such as necrosis or inflammatory cells and dye penetration into the stroma (free or engulfed in macrophages).

The results of the safety data and information from previously published series were reported to the ethics committee and permission was granted to extend testing to participants with smaller lesions. Participants were asked about pain or discomfort, and we evaluated the cornea at the slitlamp for punctate epithelial changes.

Accuracy Study
Staining was recorded using a 5-point system: none, equivocal (if it was too pale to be sure there was staining), pale blue, mixed pattern (pale and deep blue), or deep royal blue. For the purpose of analysis, any blue staining was considered positive and equivocal staining excluded from the analysis. A stratified analysis by degree of staining was also conducted. Because it would be unlikely that a clinician would be in doubt about the likely diagnosis in patients with large orbital tumors, orbital cases were excluded from this analysis. Staining (positive vs negative) was compared with histopathology (OSSN vs not OSSN).

Interobserver Variation Study
The eye was photographed before and about 30 seconds after staining for subsequent independent grading of the staining pattern. A pair of photographs was taken, one in primary gaze and the other with the lens in the center using a Nikon D90 digital camera with 105-mm lens.

Six final-year residents in the Department of Ophthalmology, University of Nairobi at Kenyatta National Hospital were trained by one author (S.G.) using projected slides showing different degrees of ToB staining. They were informed that previous studies suggested that, generally, OSSN stained positive and benign lesions were negative, but this may not be invariably the case. A week later, the same group independently assessed photographs from the last 100 consecutive participants enrolled into the study from 1 center. Cases with features that were highly suggestive of malignancy, such as very large tumors invading the orbit, were excluded. The trainer (S.G.) projected the images on a screen. None of the slides had been shown in the training session. The residents were masked to the diagnosis and did not discuss the cases. They were asked to grade the staining and suggest a diagnosis (OSSN vs non-OSSN), taking into account the clinical features of the lesion. The clinical case-mix in this sample of patients was comparable with the whole data set that included patients from all 4 study centers.

Statistical Analysis
Data were managed in Access (Microsoft Windows 2010) and transferred into Stata version 12.1 (StataCorp) for analysis. Sensitivity, specificity, and predictive values of ToB vital staining were computed based on subsequent histological diagnosis.

For the interobserver component, the scores for each clinician were compared with a reference standard using the κ statistic and graded using the Landis and Koch method.15 The examiners’ staining score was compared with the lead author’s (S.G.) assessment, while their clinical diagnosis was compared with the histopathology report. The proportions they scored as positive or negative for stain and OSSN or non-OSSN for diagnosis were reported. To calculate an average value, the κ statistics for each grader were transformed to z scores using the Fisher z transformation, averaged, and then back-transformed to a κ statistic.

Results
A STARD diagram is shown in eFigure 1 in the Supplement. A total of 537 participants with conjunctival lesions were recruited to the larger OSSN project and 447 (83%) underwent ToB staining. There were 90 people recruited into the larger study while awaiting completion of the initial ToB safety phase. The final analysis consisted of 419 participants whose median age was 37 years (interquartile range, 32-45 years) and 278 (66%) were female. There were 143 OSSN (34%) and 276 non-OSSN (66%) lesions (Table 1).

Safety Study
Seven participants with very large tumors (all were squamous cell carcinoma) were enrolled in the pilot toxicity study. None showed evidence of corneal toxicity on histology. Seven participants of the 419 (1.7%) had a mild superficial punctate keratopathy around the lesion after vital staining possibly due to disruption of the tear film by the raised lesion and the associated drying. These were distributed as follows: 4 pterygium, 1 carcinoma in situ, 1 moderately differentiated squamous cell carcinoma, and 1 capillary hemangioma. Most participants tolerated the stain well; 88 of 419 (21%) reported some mild discomfort immediately after application, all of which resolved rapidly.

Accuracy Study
Different patterns and intensities of ToB staining were seen (Figure). The 7 orbital tumors in the safety phase all stained deep royal blue. Two participants of 419 showed equivocal staining and were removed from the analysis. One had moderate intraepithelial dysplasia and the other a nevus.

Overall, 322 of 417 smaller lesions stained with ToB (77%), and staining was more frequent in the OSSN group (Table 2). Any blue ToB staining had a high sensitivity (92%; 95% CI, 87%-96%), low specificity (31%; 95% CI, 25%-36%), high negative predictive value (88%; 95% CI, 80%-94%), and low positive predictive value (41%; 95% CI, 35%-46%) compared with histology (Table 3). The low specificity was attributable to a high
Deep royal blue staining demarcated the extent of the lesion well. A mixed staining pattern was observed in which only parts of the lesion would stain, particularly actinic keratosis (Figure, F). Mucus discharge also stained blue and should ideally be wiped away before staining. Also, 133 of 275 benign lesions (48%) had leukoplakia, which stained blue. Brown pig-
mentation was found in 194 lesions (46%). These included 12 cases of conjunctival nevi. Pigmentation made interpretation of staining more difficult (Figure L).

Interobserver Variation Study
Staining results were easy to interpret. The scores of the 6 graders were similar to the lead author's (S.G.) (agreement, 91.3%) (Table 4). The lead author (S.G.) found 79% of the lesions stained with ToB compared with an average of 76.5% for the 6 graders. The average κ for staining scores was substantial (κ = 0.76). The 6 graders scored more lesions as OSSN compared with histopathology (53% vs 32%). The average κ for diagnosis was moderate (κ = 0.40).

Discussion
To our knowledge, this is the largest study to date to evaluate ocular surface vital staining for the diagnosis of OSSN. It confirmed findings from earlier studies that topical ToB
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Original Investigation Research

ever, our study indicated that the sensitivity and specificity observed.19

minutes. Washout time was rapid and no toxic effects were observed.19

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dark blue staining seen with the 0.05% preparation was similar to 1% solutions reported in other studies in South Africa and Brazil.

There were minimal adverse effects of vital staining. The mild superficial punctate keratopathy we observed may have been attributable to dry eye due to disturbance of the tear film by the raised lesion. Dry eye is the most common ocular surface manifestation of human immunodeficiency virus (HIV), with a prevalence of up to 54%.16,17 The mild discomfort reported on application of ToB may be aggravated by dry eye syndrome. The use of topical anesthetic before vital staining may prevent this. Safety studies in animals found that intraocular injection (as opposed to topical use) of 1% and 2% ToB caused irreversible damage to all the corneal layers: 0.5% damaged the stromal keratocytes and corneal endothelium but 0.25% stained the lens capsule and did not damage any corneal layer or the trabecular meshwork.18 Wander et al19 conducted animal safety studies in rabbits and guinea pigs by applying eyedrops of 0.01%, 0.1%, 0.25%, 0.5%, and 1.0% ToB to stain corneal epithelial cells. The cells picked up the vital dye within 5 minutes. Washout time was rapid and no toxic effects were observed.19

The diagnostic accuracy results of our study were similar to the ones from Brazil and South Africa showing high sensitivity and only low to moderate specificity (Table 5).11,12 However, our study indicated that the sensitivity and specificity may not be quite as high as the 2 earlier, smaller studies had suggested. However, from the clinical standpoint, the measure of accuracy that is more important than sensitivity or specificity is the predictive value. The positive predictive value in our study (41%) was lower than the South African (60%) and Brazilian (73%) studies. A caveat to such comparison is that the estimates of predictive values are only valid for the actual study population and similar populations with the same disease prevalence.

The South African study had a similar patient profile to ours with regard to age, sex, and HIV infection.12 However, there were some key differences: they used methylene blue 1% dye and had a higher proportion of OSSN. Importantly, they combined the CIN lesions with benign lesions in their analysis, however, in the analysis that was presented, conjunctival intraepithelial neoplasia was combined with benign lesions for the calculation of the test parameters.

0.05% is not associated with any significant adverse effects and found that most OSSN tumors stain.11,12 The intensity of dark blue staining seen with the 0.05% preparation was similar to 1% solutions reported in other studies in South Africa and Brazil.

The differences observed in the test performance between these 3 studies could have a number of explanations. First, it may reflect the larger sample size. There could be differences in the grading systems for describing the staining in the Brazilian and Kenyan studies were similar. However, in the analysis that was presented, conjunctival intraepithelial neoplasia was combined with benign lesions in their analysis. They did not report how the CIN cases stained or whether there were different patterns of staining (pale or mixed). The patients in the Brazilian study were older than the Kenyan and South African study participants and were predominantly male (62%). Their HIV prevalence was not reported. This probably reflects different patterns of disease. The classification of OSSN and the grading systems for describing the staining in the Brazilian and Kenyan studies were similar. However, in the Brazilian study, the concentration of ToB (1%) was 20 times that used in Kenya.

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| Table 4. Interobserver Agreement for the Evaluation of ToB Staining in 100 Patients |
| Feature | Reference Standard, No. (%) | Median, % | Agreement | Average κ (95% CI) |
| Staining resultb | 6 Graders | | |
| Positive | 79 (79.0) | 76.5 | 91.3 | 0.76 (0.68-0.82) |
| Negative | 21 (21.0) | 23.5 | | |
| Diagnosi | OSSN | 32 (32.0) | 53.0 | 70.7 | 0.40 (0.31-0.48) |
| | Non-OSSN | 68 (68.0) | 47.0 | | |
| Abbreviations: OSSN, ocular surface squamous neoplasia; ToB, toluidine blue. b The reference standard for staining was the lead author (S.G.). |

| Table 5. Comparison of This Kenyan Study With Studies From Brazil11 and South Africa12 |
| Parameter | Kenya | South Africa | Brazil |
| Vital stain dye | Toluidine blue 0.05% | Methylene blue 1% | Toluidine blue 1% |
| No. of participants | 419 | 75 | 47 |
| Female, No. (%) | 278 (66) | 45 (60) | 18 (38) |
| Age, median (IQR), y | 37 (32-45) | 35a | 58b |
| OSSN prevalence by histopathology, No. (%) | 142 (34) | 33 (44)c | 27 (57) |
| Sensitivity (95% CI) | 92 (87-96) | 97 (85-100) | 100 (87-100) |
| Specificity (95% CI) | 31 (25-36) | 50 (36-65) | 50 (27-73) |
| Predictive value (95% CI) | | | |
| Positive | 41 (35-46) | 60 (47-72) | 73 (56-86) |
| Negative | 88 (80-94) | 95 (78-99) | 100 (69-100) |
| Abbreviations: IQR, interquartile range; OSSN, ocular surface squamous neoplasia. |

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Toluidine blue has higher sensitivity and specificity for oral cancers than we observed in our study. A Cochrane systematic review showed variable sensitivity of 50% to 97% and a more uniform specificity of 98% to 99%. However, the prevalence of disease varied widely (1.4% to 50.9%). The difference, purity, and stability of ToB may have changed over time once a bottle was opened. A rising concentration may be toxic given its cell nucleus interaction and wider variety of non-OSSN lesions than the other 2 studies. Second, in the analysis using 2 × 2 contingency tables, staining was treated as a dichotomous variable, while in fact there are different degrees of intensity of blue staining. There was a high frequency of lesion pigmentation in this population but the use of more concentrated ToB may be toxic given its cell nucleus entry and may increase the false-positive rates by staining more benign lesions.

If a test has a high sensitivity, a negative result has a high chance of ruling out the disease. Toluidine blue had a high sensitivity so a negative result makes OSSN unlikely but does not completely rule it out.

Conclusions

Toluidine blue staining is safe and easily interpreted by different observers. Very few OSSN lesions did not stain with ToB. If ToB staining is negative, then OSSN is unlikely. Positive staining demarcates conjunctival lesions well, which could help in delineating the surgical excision margin, particularly for circumlimbal OSSN lesions where both the corneal and conjunctival extents would otherwise not be clearly seen. Staining also detected small recurrences of OSSN (eFigure 2 in the Supplement). Toluidine blue vital staining would not replace histopathology. The high sensitivity and low specificity make ToB a good screening tool where there is an important penalty for missing a disease. In populations with limited histopathological services, an algorithm combining ToB staining with other clinical features may raise the composite specificity for OSSN.

REFERENCES

Ocular Surface Squamous Neoplasia
From Blue Skies to Blue Dyes—We Still Need Our Ophthalmic Pathologists

Carol L. Shields, MD; Jerry A. Shields, MD

Without question, ocular surface squamous neoplasia (OSSN) is a solar-related condition, particularly endemic in immunosuppressed patients. This viral-related tumor of the conjunctival surface epithelium tends to occur in sun-exposed regions of the eye, most often at the nasal or temporal limbus. This tumor can have protean clinical manifestations as a gelatinous, translucent, foamy, leukoplakic, vascular, or pigmented mass. Risks for tumor growth onto the cornea, into the fornix, and rarely into the orbit, producing ultimate risk for metastatic disease, are understood. Treatment paradigms have shifted over the past 20 years from exclusive surgical removal using the “no-touch” technique with superficial corneal epithelectomy and conjunctival cryotherapy to surgical or nonsurgical strategies using topical antitumor medications such as mitomycin C, 5-fluorouracil, interferon alpha-2b (also available as injection), cidofovir, photodynamic therapy, and even more curios methods, including topical aloe vera. The goal of therapy is complete eradication of this low-grade malignancy to prevent recurrence, orbital invasion, metastatic disease, and death.


Invited Commentary

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