

Toluidine blue: A review of its clinical utility

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ABSTRACT: *The first and foremost duty of an Oral Pathologist or any general dentist is to detect a premalignant lesion sufficiently early to prevent their progress to invasive carcinoma. Though final diagnosis always should be given on the basis of laboratory-based investigations which sometimes are associated with variable false-negative rates, but Toluidine blue vital staining can serve as an important chair side investigation in detecting prompt premalignant lesion. Its feasibility rate is high since it is simple, inexpensive and sensitive in nature. Chemically Toluidine blue is a basic thiazine metachromatic dye having high affinity for acidic tissue components like DNA and RNA thus serves in detecting tissues rich in former mentioned proteins. Its metachromatic property leads its wide applications both as vital staining in living tissues and as a special stain. So it is being used often in vivo to identify dysplasia and carcinoma of the oral cavity. Its aim is to highlight components, such as mast cells granules, mucins, and cartilage. Thus this article will shed some light on principle, basic technique, its necessity and the various applications of toluidine blue.*

KEYWORDS: *Toluidine blue, Metachromasia, neoplastic cells, chromotropes*

INTRODUCTION:

Toluidine blue [TB] also known as tolonium chloride is a characteristic acidophilic metachromatic dye that selectively stains acidic tissue components like sulfates, carboxylates, and phosphate radicals^[1] and thus binds to tissues rich in high DNA and RNA content^[2]. Toluidine blue has been discovered by William Henry Perkin in 1856 after which is being extensively used as a vital stain for mucosal lesions and also has been applied in various tissue sections in order to specifically stain certain components owing to its metachromatic property. TB was first applied for in vivo staining by Reichart in 1963 for uterine cervical carcinoma in situ^[3]. TB detects relative rather than absolute differences between normal and malignant cells and tissue. They are available as 1% or 2% oral rinse either in aqueous form or as weak acid solution or of undefined formulation. Only about 5% of dye by weight is retained in oral cavity following expectoration^[4]. Application of TB in vivo is based on the fact that both dysplastic and neoplastic cells should supposedly contain more nucleic acids quantitatively than normal tissues. Malignant epithelium may also possess intracellular canals that are wider than the normal epithelium, thus facilitating penetration of the dye^[1]. Vital staining of the oral epithelium can be a method of surveillance in high risk patients to oral cancer and for those who had confirmed neoplasms of other parts of aero digestive tract^[4]. TB has been used as a vital stain to highlight potentially malignant oral lesions and can be useful in detecting early lesions, which could be missed out on clinical examination. Moreover, it can outline the full extent of dysplastic epithelium or carcinoma prior to excisions^[5] and can detect multicentric or second tumors and can help in the follow up of patients with oral cancer. TB helps in obtaining the marginal control of carcinoma and in selecting the biopsy sample site in premalignant lesions. TB-stained tissue may appear dark royal blue or pale royal blue color^[6]

TISSUE STAINING:

PRINCIPLE: Based on the principle of metachromasia tissue staining is mainly done by TB. The dye has the ability to produce a different color from that of the original dye on coming in contact with the tissues. In 1875 by Cornil, Jurgens, and Ranvier discovered metachromasia. It is important because of its highly selectiveness and only certain tissue structures can stain metachromatically. Metachromasia is a phenomenon by which a dye may absorb light at different wavelengths based on its concentration and surroundings and has the capability to change its color without tampering its chemical structure. The physical changes are a specialized, orderly form of dye aggregation that brings about this color change. For metachromasia to occur there must be free electronegative groups on the surface of tissues^[7]

Metachromasia is attributed to stacking of dye cations at the sites of high density of anionic groups within the tissue. Stacking shortens the wavelength of maximum absorption, a hypochromic shift, in order that the utmost wavelength within the spectrum of the transmitted light is longer making the observed color red instead of blue^[8]. Substances like mucins, mast cells etc are having the capability to stain in the above mentioned ways thus they can be called as chromotopes ^[9]. The chromotropes carry acidic groups with a minimum density of less than 0.5 nm between adjacent charged groups. These alter the color of metachromatic dyes. Principally, in order to make dimers, trimers, or polymers, van der Waals force come into being to bind the dye together. Other forces like hydrogen and hydrophobic bonding also exist sometimes but mostly they play a lesser role. The dye exists during a normal monomeric or orthochromatic form to a polymeric or metachromatic form. The chromotropes are negatively charged which in turn attract the positively charged polar groups on the dye resulting in formation of dye-to-dye cluster in a specialized orderly way forming a polymeric form. Metachromasia can be seen in three main forms namely alpha (α), beta (β), and gamma (γ) giving a range of colors^[Table 1] ^[10]

TYPE	COLOR	STRUCTURE	RESULTS
α - ORTHOCHROMATIC	BLUE	MONOMERIC	NEGATIVE
β -WEAKLY METACHROMATIC	PURPLE	DIMER AND TRIMER	WEAKLY POSITIVE
γ -STRONGLY METACHROMATIC	RED	POLYMERIC	STRONGLY POSITIVE

[TABLE-1]

The color shift is usually from a blue or violet dye to yellow or red stain meaning that the colour absorption shifts to shorter wavelengths, leaving only the longest wavelengths to be seen. This is often believed to represent polymerization of the dye. The greater the degree of polymerization, the stronger is that the metachromasia. Water is required in between the dye molecule in the process of metachromasia in order to form the polymer and doesn't survive dehydration and clearing^[9]. The spectrum of TB with an orthochromatic tissue appears maximum at about 630 nm and marking result is blue. With a metachromatic substance the spectrum is maximum at 480–540 nm and therefore the staining is red in color ^[10]. The affinity seen while binding to DNA or RNA are mainly due to the Van der Waals attraction between Toluidine blue and subsequent polyanions which is also similar in case of hydrophobic bonding. However, flexible, high charge density glycosaminoglycans (GAG) permit charge neutralization of dye aggregates or stacks formed thanks to dye-to-dye van der Waals attractions and hydrophobic bonding ^[8]

NEED FOR CLINICAL APPLICATION OF TOLUIDINE BLUE:

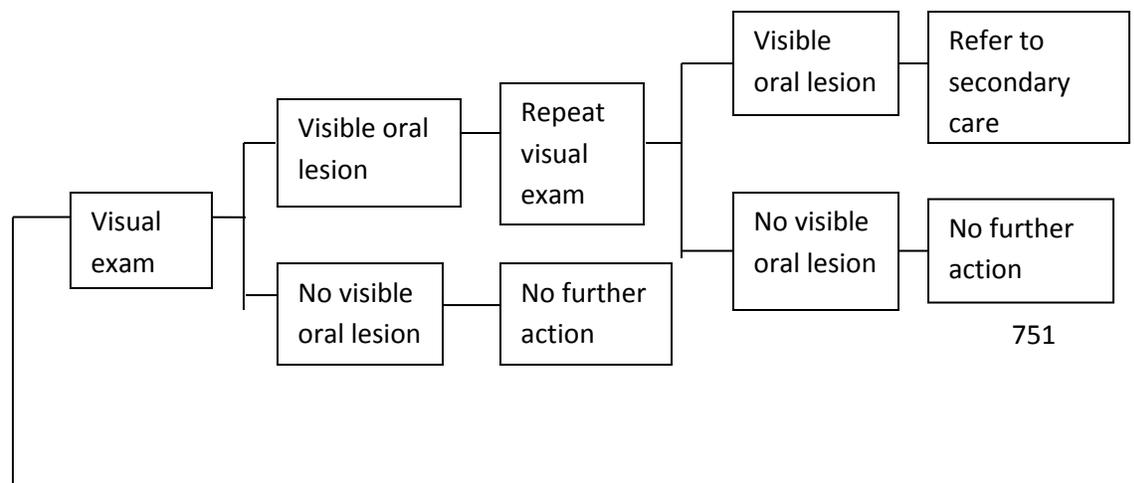
Prognosis of oropharyngeal squamous cell carcinoma (SCC) (oral cavity and pharynx) depends on early diagnosis, despite advanced surgical techniques and adjuvant treatment, the 5-year survival rate remains ~40-50% ^[11,12]. Unfortunately, most of the time patients come with complaints to dentists when it is symptomatic and, minimum two thirds of the patients present with an advanced stage. Treatment for this requires gives rise to a high

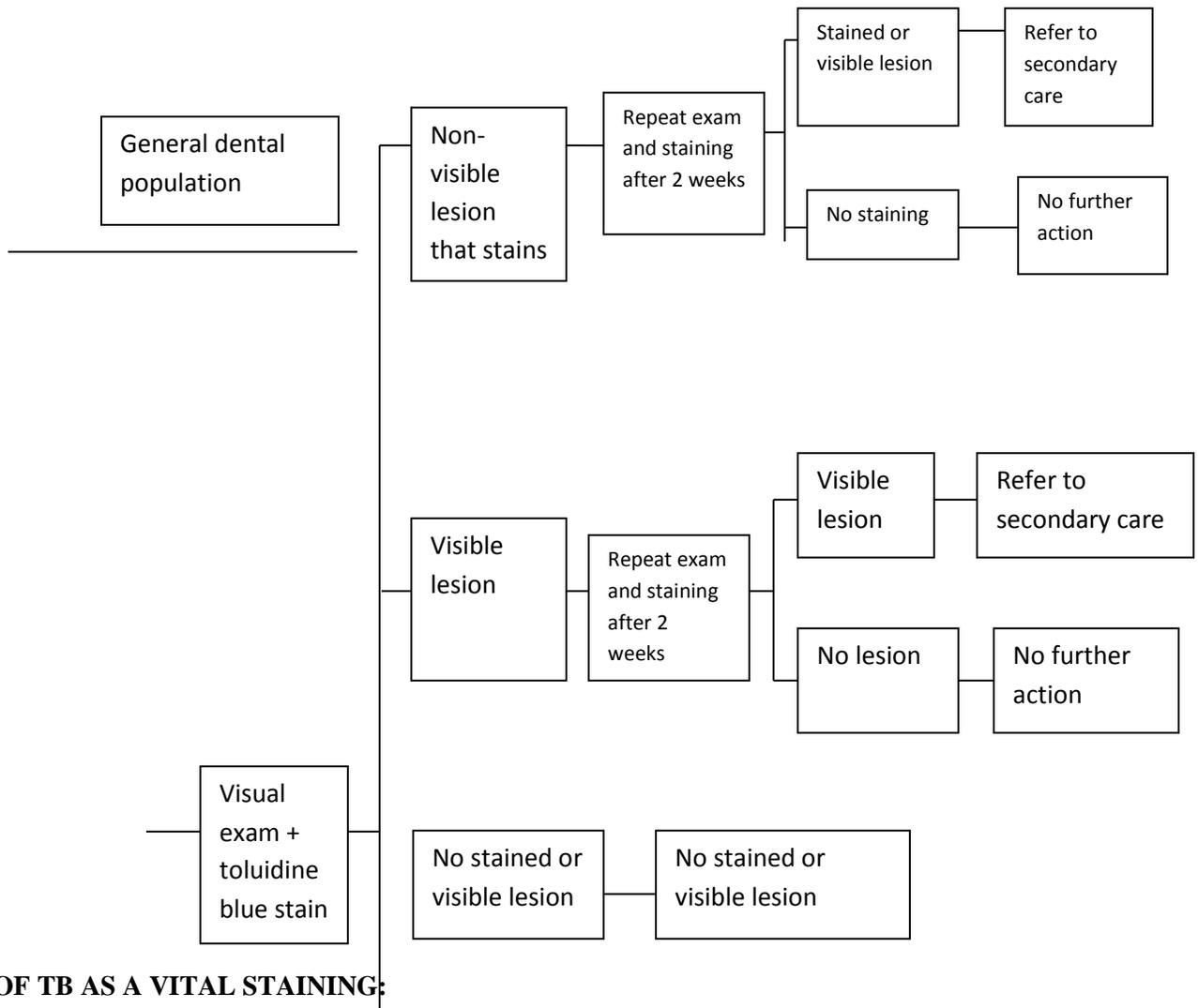
rate of morbidity and mortality and, moreover, early detection of oro-pharyngeal pre-malignant lesions is required to improve the survival rate and quality of life (QoL). In many cases, it is seen that clinicians find it difficult to recognize patients at high risk of developing oral cancer that is mainly because of specific time and site of the biopsy taken from suspected lesions and this largely depends upon the clinical ability of that general practitioner to differentiate pre-malignant lesions from reactive and inflammatory diseases. This difficulty arises even more when there is a typical patch or plaque that appears clinically as leukoplakia [13]. In a lesion that appears as a leukoplakia, in 16% of the cases, the lesion is already malignant [14,15] while in the dysplastic leukoplasic lesions, the risk of cancer development has been reported to be as high as 43% [16] within a mean time of 8.1 years. Various techniques have been developed to verify clinical examinations, with the aim of improving early oro-pharyngeal cancer diagnosis. Since dysplasia and in situ carcinoma contain increased DNA and RNA than the normal surrounding epithelium, the use of in vivo staining, by toluidine blue dye, depends on the fact that it is an acidophilic dye that selectively stains acidic tissue components like DNA and RNA. Toluidine blue staining is considered to be sensitive in identifying early oro-pharyngeal premalignant and malignant lesions [17-21].

APPLICATION OF TB:

- When applied to connective tissue mucins, especially acid mucins stain turns purple to red, while the background stained blue.
- Presence of Heparin and histamine in Mast cell granules results purple colored staining.
- Amyloid on the other hand shows characteristic bright red birefringence but under polarized light it appears blue.
- When TB of concentration 0.01% is used on endocrine cell granules they give purple to red stain.
- Sulfatides appear in red brown or yellow stain. Only acidic lipids sufficient enough to induce a metachromatic shift are stained.
- Corneybacterium diphtheria gives red violet color on staining since the granules contains polymerized inorganic polyphosphate.
- When Helicobacter is stained with 1% concentration TB, it gives off deep blue color against a variably blue background.[23]
- The rapidity of the staining procedures(10–20 s) and better clarity of cells by TB staining can be of help to stain frozen section.

DECISION TREE: Comparison between an oral cancer screening in general dental practice both with and without the use of toluidine blue as an adjunct to clinical examination has been described in the following decision tree with the help of flowchart in the figure 1.. It is a simplification in that it only extends as far as diagnosis; some of those patients with a diagnosis of dysplasia or pre-cancer may pass through the decision tree on more than one occasion, others may receive treatment [22].





UTILITY OF TB AS A VITAL STAINING:

Richart first proposed the use of TB as vital stain to detect dysplasia and carcinoma in situ of the uterine cervix followed by Neibel, Chomet, Shedd and co-workers were first of all to report the importance of vital application of TB for the detection of premalignant and malignant lesions of the oral cavity. They only confirmed the property of TB to confirm clinically suspicious lesions as neoplastic, to detect properly the boundaries of premalignant and malignant growth, and to identify any unnoticed or satellite tumors ^[24] Main principle behind the usage of TB as chairside investigation is because of the fact that more nucleic acids in dysplastic and neoplastic cells will lead in TB uptake by the tissues since it has high affinity towards acidic components. The other proposals regarding the uptake of TB stain in dysplastic and carcinomatous lesions include the high density of nuclear material, loss of cell cohesion, and increased rate of mitosis ^[6] The use of TB as a successful supporting diagnostic aid in the identification of malignant lesions has resulted in publishing numerous studies, though many of them unfortunately came out with diverse results. The diversity could be due to different reasons, it could be because of variations in methodology, the particular population on which the studies are done, and type of lesions analyzed. In addition, according to some studies, biopsies of the lesions that did not retain

Toluidine Blue were not performed. These factors may have an interference with the proper evaluation of staining, thus limiting the value of the results obtained [25]

PREPARATION AND METHOD OF APPLICATION:

Generally for oral use 1% concentration of TB is prepared. A 100 mL of 1% TB is prepared with 1 gm of Toluidine Blue powder, taking 10 mL of 1% acetic acid, absolute alcohol 4.19 mL in quantity, and 86 mL distilled water. The pH is usually regulated to 4.5 [26]. The technique of application usually involves rinsing of the mouth twice with water for 20 s to remove debris followed by application of 1% acetic acid for 20 s in order to remove ropey saliva. Then 1% TB is applied for 20 s either with cotton swab in presence of any mucosal lesion or given as rinse when no obvious lesion was detected. Again, to reduce the extent of retention of stain mechanically 2 rinses with 1% acetic acid were performed. Finally the mouth is rinsed with water [26] THE

INTERPRETATION: It is based on the color; a dark blue (royal or navy) stain is considered positive, light blue staining is doubtful and when no color is observed, it is interpreted as negative stain. Under normal conditions, nucleated scales covering the papillae on the dorsum of the tongue as well as the pores of seromucinous glands in hard palate are frequently stained with TB [27]

SENSITIVITY AND SPECIFICITY: Over the years various studies have been performed in order to determine the sensitivity and specificity of in vivo TB staining. Contrary with the previous studies which concluded that range of sensitivity of Toluidine Blue to be 86–100% and specificity be 44–100% [26,28–30], Lingen et al, [5] in their review, mentioned the sensitivity and specificity of TB to be in the range of 78–100% and 31–100%, respectively regarding the detection of oral cancer. During the analysis of capability of TB staining on identifying oral premalignant lesions and carcinoma in animal models showed high false-negative results in premalignancies (95.2%) seeding doubt on whether TB is sensitive enough for the detection of premalignant lesions. In contrast, it was found that TB when stained on in vivo condition, shows high sensitivity in detecting carcinoma. In 1989, meta-analysis of available data assessing the effectiveness of TB application in identification of oral squamous cell carcinoma determined sensitivity ranging from 93.5% to 97.8% and specificity ranging from 73.3% to 92.9% [31] Epstein et al [1] showed sensitivity and specificity of 92.5% and 63.2%, respectively, in their results [24]. When 145 oral mucosal lesions analysed out of 102 patients, in vivo stain of Toluidine Blue revealed 100% sensitivity and 62% specificity. So, TB staining having a remarkable sensitivity in the detection of invasive carcinoma was considered to be an irreplaceable option in surveillance of high-risk subjects. The study also discovered that lesions with little dysplasia or atypia stain irregularly with Toluidine Blue. Reason behind the 20.5% false-negative results exhibited by oral premalignant lesions could be due to absence of objective criterias for evaluation of stain uptake [4]. When Toluidine Blue was applied in 81 lesions of which clinically 48 lesions appeared suspicious and 33 benign showed that 28 cases did not uptake any stain, 20 had equivocal stain and 33 stained positively. Biopsy report of these specimens revealed 54 to be nonmalignant and 27 malignant. The study concluded with 100% sensitivity and 52% specificity [2]. The study done by Onofre et al [25] on TB staining in premalignant lesions, and superficial oral ulceration suggesting malignancy showed 100% sensitivity of it in the detection of in situ and invasive carcinoma and no false-negative results came out. The lesions suspected as dysplasia did not successfully retain the stain, and thus gave false-negative results. It could be since the exact procedure by which the dye differentially stains malignant or dysplastic tissues remain unknown. Staining specificity was 65% because of the successful exclusion of inflammatory lesions in the first time and was re-stained after 14 days. Specimens without epithelial dysplasia or atypical cells, rate of false positivity showed 35%. Hegde et al [27] in their study founded a sensitivity of 97.29% and specificity of 62.5% where as false positivity of 7.69% and false negativity of 16.67% in the result. The authors suggested that specificity was decreased due to retention of the dye in some benign lesions. Gupta et al [32] demonstrated sensitivity of 96.9% and specificity of 86% for detection of malignancy. The false-positive rate reported was 14%, etiology could be ulceration, inflammation, or traumatic lesions. The study too reported about 64% sensitivity and 86% specificity for premalignant lesions. False-positive retention in non-dysplastic conditions has been reported with varying frequency. Mashberg [26] reported a false-positive rate of 8.5%. There may be a chance to reduce false positivity

with the help of second examination done typically 14 days after which in turn help in excluding pre stained lesions due to inflammation.

In vivo TB staining might be the result of immediate binding of sulfated mucopolysaccharides, which are found in higher quantity in tissues that are actively growing, such as tumors and tissues that are healing which may explain false-positive results [29]. A study by Martin et al [33] showed 42% false negative for in situ carcinoma and 58% false negativity in epithelial dysplasia. This could be due to the sample analyzed and the fact that these authors considered only lesions with stippled or dark blue staining as positive.

DRAWBACKS:

1. Problems with studies of Toluidine Blue included that those were not carried out in a primary care environment
2. So far the data what received from studies are all done in secondary care so they are not necessarily applicable to general population,
3. No studies have been performed on randomized controlled trials, few studies only take carcinoma or dysplasias in inclusion factors, and some may include both without any uniformity.
4. Rarely histological diagnosis has been used as the “gold standard,” and no single standard staining method used resulting in establishing confusion regarding inclusion of pale staining as positive or negative [5]

CONCLUSION:

TB application is an important adjunct to the clinical examination because it may not only increase the clinical suspicion of the examination but also assist in identifying sites in need of biopsy and delineating margins of the lesions, which may lead to a more timely diagnosis allowing benefits of earlier treatment and in directing surgical management [34]. Although the specificity of the technique is less which could be attributed to the inability to biopsy normal tissues which does not take up TB, it can still be used as an important adjuvant as a diagnostic aid because in a clinical setting, false-positive results are of less concern than false-negative results and any positive findings should be confirmed on biopsy for the presence of dysplasia or carcinoma.

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