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Toluidine Blue Stain as a Rapid Onsite Tool for Preliminary Diagnosis in Imprint Smears of Bronchoscopic Biopsy: A Cytohistopathological Correlation

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Abstract

Introduction Lung cancer is currently the most common cause of cancer-related mortality, with 11.4% of cancers and 18% of cancer-related deaths worldwide whereas Indian figures are 6.9 and 9.3%, respectively. Hence, the need for early diagnosis. Bronchial biopsy has the highest sensitivity among all the samples that can be obtained by bronchoscopic techniques in case of endobronchial lesions. Imprint cytology has emerged as an important cytological method. Toluidine blue has been studied for its use as rapid onsite stain for cytological evaluation on various samples of cytology in different anatomic sites. This has helped in quick and less expensive, preliminary reporting.

Objectives This article aims to assess the efficacy of onsite toluidine blue stain on imprint smears of bronchoscopic biopsies to diagnose malignancy in suspected cases of lung carcinoma.

Study Type Prospective study on accuracy of a diagnostic test.

Materials and Methods A total of 100 cases of bronchoscopy were included in the study. The patients were clinico-radiologically suspected to have brochogenic carcinoma and all of them were subjected to biopsy. Imprint smears were prepared from the bronchoscopy biopsy specimens. Smears were stained onsite with toluidine blue stain, and histopathology sections were stained with hematoxylin and eosin, also confirmed by immunohistochemistry.

Results Sensitivity and specificity of onsite toluidine blue stain for malignancy reporting were 97.9 and 80%, respectively, when compared to histopathology as standard.

Conclusion Toluidine blue can be used as an onsite staining method on imprint smears of bronchoscopic biopsy for preliminary detection of lung malignancy.

Keywords
- bronchoscopic biopsy
- cytohistological
- lung carcinoma
- toluidine blue

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Introduction

Lung cancer is currently the most common cause of cancer-related mortality, with 11.4% of cancers and 18% of cancer-related deaths worldwide whereas Indian figures are 6.9 and 9.3%, respectively. Hence, the need for early diagnosis[1] There are several methods to obtain samples from suspicious lung masses. Bronchial biopsy has the highest sensitivity among all the samples that can be obtained by bronchoscopic techniques in case of endobronchial lesions.[2] In several studies conducted worldwide, imprint cytology has come up a long way as a cytological method, to improve diagnostic accuracy as it provides viable tumor cells directly from the lesions. This has helped in quick and less expensive, preliminary reporting.[3–9]

Different stains can be applied to imprint cytology samples for cytology analysis. Toluidine blue stain (TBS) is a basic metachromatic dye with high affinity for acidic components like nucleic acids and therefore binds to nuclear material of tissues with a high deoxyribonucleic acid and ribonucleic acid content.[10] TBS has been extensively used by various researchers as a vital stain for oral mucosal lesion and aspiration cytology reporting in recent years.[11–13] The literature shows, TBS is a practical, rapid, inexpensive, and effective adjunct diagnostic tool. It is a supravital stain that accentuates good cytological and nuclear details and enables the three-dimensional view of cells in wet mount film. It is easily available, very cheap, cost-effective, and used for quick reporting.[12]

Many of the studies with TBS were done on fine-needle aspiration cytology (FNAC) and oral mucosal lesions. TBS has also been used earlier by several researchers in their study for Rapid Onsite Evaluation (ROSE) on different sites like lymph nodes, thyroid, breast, and salivary glands.[12] In case of lung, it has been used in transthoracic needle aspirations (TTNAs).[14] But there is no study to the best of our knowledge showing the use of TBS as an onsite staining procedure for imprint smears of bronchoscopic biopsy. Hence, we undertook this study on imprint smears, to see the efficiency of TBS, comparing it with histopathology taken as gold standard.

Objective

To assess the efficacy of onsite TBS on imprint cytology of bronchoscopic biopsies to diagnose malignancy in suspected cases of lung carcinoma.

Materials and Methods

This study was a prospective one, conducted in the department of pathology and department of pulmonology of a teaching institute, after prior approval from institutional ethics committee. The specimens for cytological and histological examination were collected from the indoor and outdoor patients of the pulmonology department in whom a provisional diagnosis of lung carcinoma was made according to clinical and radiological findings. A total of such 100 patients were included in the study. Patients who were not willing to undergo the procedure or were not medically fit for the procedure were excluded. The samples were obtained by flexible fiberoptic bronchoscopy done by the pulmonologist. Imprint smears were prepared in the bronchoscopy suite itself from the bronchial biopsy in all the 100 cases. Imprint smears were prepared by placing forceps biopsy specimens on a glass slide by gentle touching and rolling over the surface. Care was taken to avoid crushing the specimen. All imprints were dipped in 95% alcohol for 20 seconds and then 2 drops of TBS prepared earlier were added to the wet slide. The slide was cover slipped, excess stain blotted out, and slide was subjected to cytological examination. The TBS smears were reported by an experienced pathologist onsite regarding adequacy, and if adequate, a provisional diagnosis was given. If the imprint was found to be of scant cellularity, or hemorrhagic/necrotic material, inadequate for reporting, further imprint was taken. After making the initial onsite reporting, the slides were again dipped in alcohol for permanent cytological staining with Papanicolaou (PAP)/hematoxylin and eosin (H&E) stain. However, permanent cytological stains were not included as a part of the current study. Bronchial biopsy specimens were fixed in 10% neutral-buffered formalin, and formalin-fixed paraffin-embedded sections were prepared, which were subsequently stained with H&E. Onsite TBS cytology and histopathology slides were viewed independently by different pathologists. Immunohistochemical confirmation was done for the histopathology samples. Data were analyzed subsequently for sensitivity and specificity of TBS.

Results

Toluidine blue stained smears yielded 100% adequacy in case of reporting. All cases were grouped under two heads for onsite stained smears—"positive for malignancy" and "negative for malignancy." The sensitivity and specificity of malignancy detection with toluidine blue stained imprint smears were 97.9 and 80%. There were two false negative and one false positive malignancy cases (►**Table 1**). Out of 93 cases diagnosed as "positive for malignancy" by TBS imprint cytology, only 10 cases could not be separately subclassified into specific type. Others were grouped as "small cell" and "nonsmall cell" carcinomas (►**Fig. 1A, B**). Histopathological final diagnosis along with immunohistochemical confirmation of 100 cases yielded 95 cases to be malignant, with squamous cell carcinoma being the most common subtype (48 cases) (►**Table 2**, ►**Fig.2**), followed by small cell carcinoma (31 cases) and adenocarcinoma (16 cases) (►**Fig. 3A, B**). There were four cases of granulomatous inflammation, later found to be of tubercular etiology. History from the patients, including males and females revealed that 90 out of 95

Table 1 Results of toluidine blue stain

		Histopathology Results	
		Malignant	Nonmalignant
Toluidine blue stain results	Positive for malignancy	93	1
	Negative for malignancy	02	4
	Total	95	5

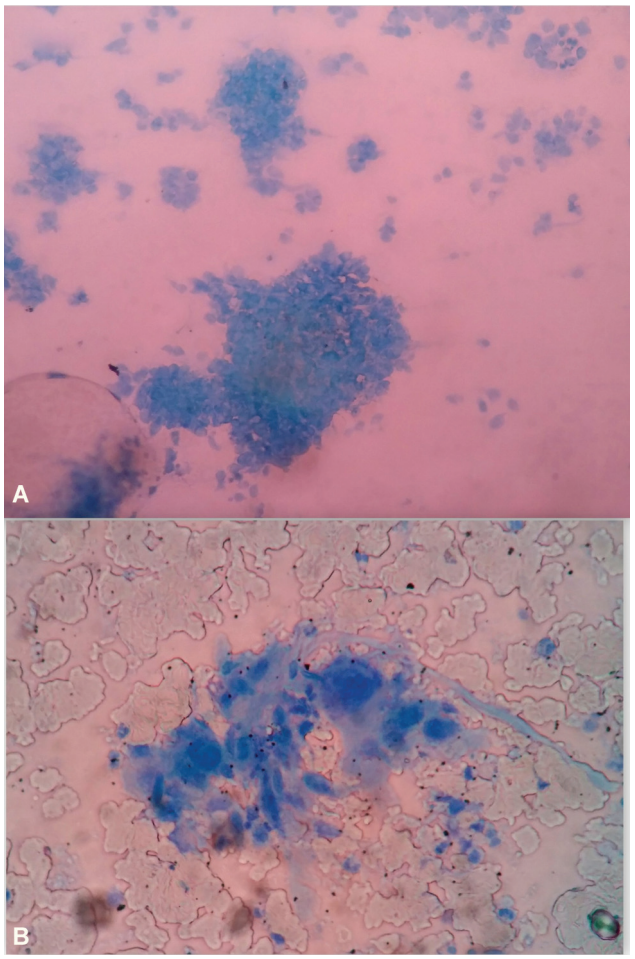

Fig. 1 (**A**) Imprint smear positive for malignancy, nonsmall cell carcinoma (favors adenocarcinoma – showing three-dimensional clusters and acinar structures, toluidine blue stain ×400). (**B**) Imprint smear positive for malignancy, nonsmall cell carcinoma (favors squamous cell carcinoma – showing atypical squamous cells, toluidine blue stain ×400).

Table 2 Histological subtypes of all cases

Histological subtype	Number of cases
Squamous cell carcinoma	48
Small cell carcinoma	31
Adenocarcinoma	16
Granulomatous lesion	4
Nonspecific inflammation with reserve cell hyperplasia	1
Total	100

malignant cases had used tobacco either in form of smoking or orally for chewing/as toothpaste. The patients' age group ranged from 41 to 75 years, and the mean age was 62 years.

Discussion

In the current study, we aimed at assessing value of toluidine blue as onsite stain for imprint smear cytology of bronchoscopic biopsy specimens in suspected cases of lung carcinoma. Our main aim was to assess the method for diagnosing malignant lesions on cytology. However, in addition, the adequacy of imprints smears was given by TBS. This helped in ensuring sufficient sample for assessment. The provisional diagnoses were compared with final histopathological diagnosis, which was used as gold standard. Our study yielded a sensitivity of 97.9% and specificity of 80% for TBS onsite stained imprint smears. Eighty-three out of 93 cases were also correctly subtyped into "small cell" and "nonsmall cell" carcinomas.

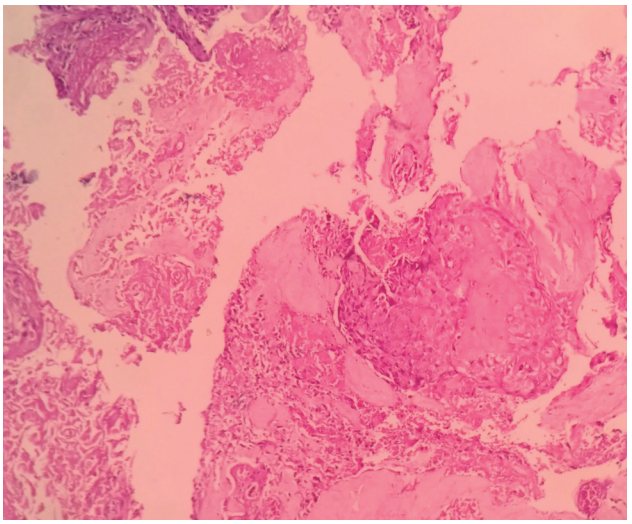

Fig. 2 Squamous cell carcinoma, histopathology (hematoxylin and eosin [H&E], ×100).

Several researchers have carried out studies on onsite staining using different stains. In a study of rapid on site staining using brilliant cresyl blue by Sofi et al for FNAC smears on several sites, majority of the cases (approximately 95%) appearing malignant with brilliant cresyl blue staining correlated with PAP and/or H&E staining.[15]

Patil and Nikumbh conducted a study to see the efficacy of aqueous TBS smears in comparison to conventional smears stained with PAP stain of cervico-vaginal smears and to reduce the reporting time of smears and also cutting down on the cost. They concluded that the TBS is better for cervico-vaginal cytology and preferred over PAP staining with respect to staining properties of nucleus as well as cytoplasm.[16]

In a recent study by Anila et al, TBS was used to investigate the value of ROSE in TTNA of patients with pulmonary nodules.[8–11] The authors concluded that ROSE can subclassify the morphological type of bronchogenic carcinoma in a majority of cases.[12] The use of ROSE helps in ensuring the adequacy of sample, minimize the number of passes required, and thereby minimize complications such as pneumothorax.[14]

Chandra et al conducted a study to assess the role of cytology in the diagnosis of lung lesions and to compare it with histopathology. It was also intended to evaluate the role of ROSE as an adjunct to cytological diagnosis of lung lesions. The study included all the cases of lung lesions, which were diagnosed on cytology followed by histopathology. Among other cytological techniques, imprint cytology was also done.

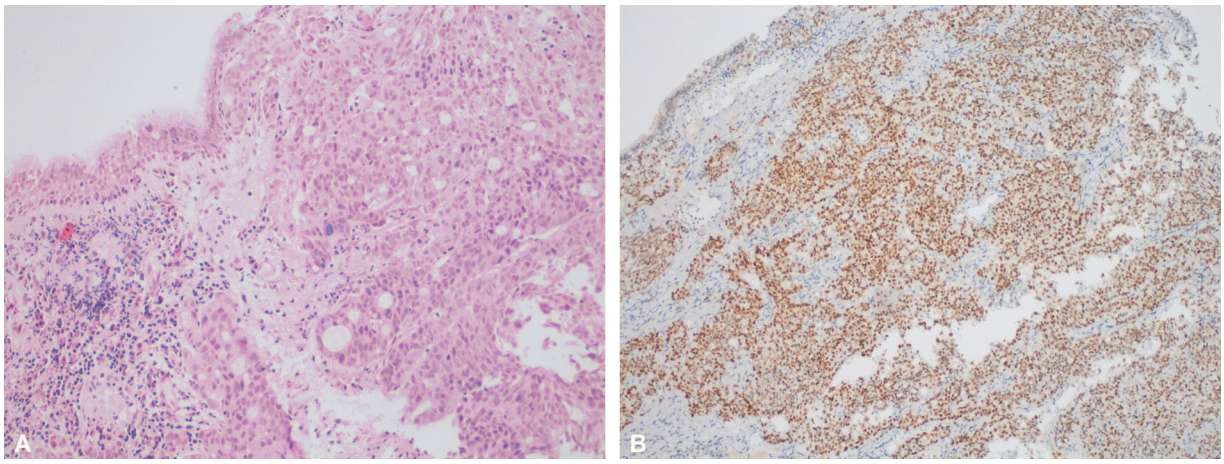

Fig. 3 (**A**) Adenocarcinoma (hematoxylin and eosin [H&E] ×200). (**B**) Adenocarcinoma, immunohistochemistry (IHC) (thyroid transcription factor 1 [TTF-1] ×100).

In addition to the adequacy, which increased to 93.4% with TBS, it was concluded that cytology is comparable to histology in the diagnosis of lung lesions and may even outperform biopsy in lung tumor diagnosis. Also, preliminary distinction between neoplastic and non-neoplastic was possible with TBS in a fair number of cases.[17]

Verma and Gupta in their study of rapid method of cytology diagnosis by supravital staining in FNAC of various tissue and organs found that out of 100 cases, 93 cases (93%) were diagnosed correctly and discrepancy of 7 cases found in cytological diagnosis by TBS and H&E stain. Non-neoplastic cases were diagnosed correctly with the overall accuracy of 93.75% and neoplastic cases were diagnosed with an accuracy of 92.30%. They concluded that the advantage of this technique was that cells are seen in living natural condition without any artifact caused by fixation, air dry, or cutting.[18]

In a study from Pakistan by Saba et al it was found that toluidine blue stained study of FNAC improves the diagnostic accuracy by minimizing the smearing and drying artifact, loss of cell sample during fixation and staining which influences the diagnostic accuracy. In their study, the diagnostic accuracy by using PAP stain was 78%, while it was up to 100% with supravital TBS smears. Moreover, the percentage of inadequacy was reduced to just 25%.[12]

In 2012, Ammanagi et al showed that onsite TBS and screening improved the efficiency of FNAC reporting.[13] Various researchers like Miller et al, Onofre et al, Mashberg, and Hedge et al showed in their respective articles TBS as diagnostic adjunct in detection of asymptomatic oral squamous cell carcinoma and its utility in early detection of oral malignancies.[19–22] Warnakulasuriya and Johnson discussed the role of toluidine blue mouth rinse in oral cancers.[23]

In his review article, Hassan has observed, ROSE has shown acceptable sensitivity both for malignant and benign disease. He also opined that the role of onsite stains is emerging in molecular testing for lung cancer, and use of telemedicine and telepathology were important aspects to propagate the application of the procedure.[24]

All the above studies were on variety of cytological specimens, providing important directions and reference to our research. Every study has opined that onsite stain such as toluidine blue can effectively classify tumors based on cytology, in addition to its indispensable role in checking for adequacy. However, there were no studies in particular with focus on imprint smears of bronchoscopic biopsy. With the importance of imprint cytology been outlined by various studies earlier, it is important to identify cost-effective, accurate, and quick methods for the same. Results from our study showed that nuclear staining and diagnostic properties of TBS is comparable to and no less than permanent stains used in earlier studies.[3–9]

However, there was one false positive case and two false negative cases in our study with TBS. Comparable rates of false positive and false negative results with PAP cytology were also reported in other studies. An earlier study by the author found similar results with Leishman-Giemsa cocktail stain.[25] False positive case may arise due to crushing artifact during biopsy acquisition or distorted cytomorphology produced during the imprinting process.[5] False negative cases might be due to the scanty tumor cells at the surface of specimen. Imprint cytology, as a method, is solely based on the amount of tumor cells the biopsy specimen provides. In cases of lepidic predominant adenocarcinoma, there is, by definition, minimal recognizable cellular atypia—hence a potential source of misjudgment.[26,27]

Although all studies have opined that onsite staining is beneficial, human resource constraints have been taken up by a group of authors. Mehrotra et al are of the opinion that the affordability of ROSE is questionable from a human resource perspective as there are availability issues with regards to the time spent providing ROSE in the bronchoscopy suite itself. This leads to unavailability of pathologists to interpret onsite stains and requirement of training of cytotechnologists for the same.[28]

Conclusion

From the current study it may be concluded that there is ample scope of using toluidine blue as onsite stain to check the adequacy and also the preliminary detection of

malignancy along with their subtyping on imprint cytology of bronchoscopic biopsies. Toluidine blue as a stain shows up very good nuclear details. Nonetheless, extensive studies are required to validate these results.

Authors' Contributions

D.C. - Conceptualized the study.
A.C. - Carried out the study and took part in writing manuscript.
R.B. - Writing and proof checking of manuscript.
J.P. - Writing and proof checking of manuscript.

Ethics Clearance

The study was cleared by institutional ethics committee, IPGMER Kolkata. Same has been mentioned in the "Materials and Methods" section of the manuscript.

Conflict of Interest

None declared.

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