Section: Pathology

Original Research Article

TOLUIDINE BLUE- A RAPID STAIN TO RECKON FOR CONVENTIONAL CYTOLOGICAL EVALUATION

Kallur Anisha Begum[1], B. Syam Sundar Rao[2], Kharidehal Durga[3], N. Mohan Rao[4]

[1]*Post graduate, Department of Pathology, Narayana Medical College, Nellore, Andhra Pradesh, India.*
[2]*Professor, Department of Pathology, Narayana Medical College, Nellore, Andhra Pradesh, India.*
[3]*Professor & HOD, Department of Pathology, Narayana Medical College, Nellore, Andhra Pradesh, India*
[4]*Professor, Department of Pathology, Narayana Medical College, Nellore, Andhra Pradesh, India.*

Received *: 04/04/2025*
Received in revised form *: 30/05/2025*
Accepted *: 17/06/2025*

Corresponding Author:
Dr. Kharidehal Durga,
Professor & HOD, Department of Pathology, Narayana Medical College, Nellore, Andhra Pradesh, India.
Email: drkdurga.path@gmail.com

DOI: 10.70034/ijmedph.2025.3.222

Source of Support: Nil,
Conflict of Interest: None declared

Int J Med Pub Health
2025; 15 (3); 1201-1208

ABSTRACT

Background: Fine Needle Aspiration Cytology (FNAC) is a minimally invasive, rapid, and cost-effective diagnostic procedure commonly used to evaluate both superficial and deep-seated lesions. This study investigates the diagnostic utility of toluidine blue, a supravital stain, as an alternative to conventional Hematoxylin and Eosin (H&E) staining, focusing on its effectiveness in rapid sample adequacy screening, provisional diagnosis, and tissue preservation.

Materials and Methods: A prospective study was carried out on 1200 FNAC samples from various anatomical sites over a six-month period. Smears were prepared and stained using different concentrations and durations of toluidine blue to determine optimal staining conditions. Cytological interpretations were compared with those from H&E-stained smears to evaluate diagnostic concordance and accuracy.

Results: The most effective staining was observed with 0.4% toluidine blue for 20 seconds. This protocol allowed clear visualization of cellular and nuclear details, enabling immediate evaluation. Toluidine blue achieved 98% diagnostic accuracy for non-neoplastic lesions and 92% for neoplastic lesions, with an overall concordance rate of 94% when compared with H&E. The technique also facilitated immediate repeat sampling when necessary, reducing turnaround time and improving tissue utilization. Minor diagnostic discrepancies were noted in certain borderline thyroid and lymphoproliferative lesions.

Conclusion: Toluidine blue is a simple, rapid, and economical staining method for FNAC smears. It provides sufficient cytomorphological detail for early diagnosis and is especially valuable for rapid on-site evaluation (ROSE) and in low-resource settings. Its implementation can enhance diagnostic workflows, reduce repeat procedures, and support timely clinical decision-making.

Keywords: Rapid Stain, Toluidine Blue, FNAC.

INTRODUCTION

Fine needle aspiration cytology (FNAC) is an accurate, cost-effective tool and an essential basic diagnostic technique to investigate superficial and deep lesions.[1,2] It remains one of the most important contributions for rapid diagnosis from practical point of view and its clinical value is not limited to neoplastic conditions.

Routinely FNA slides are stained with Hematoxylin and Eosin (H&E), Papanicoloau, and/or Giemsa for microscopic examination. For an FNA an adequate and representative sample is the key to correct diagnosis.

Rapid stain using toluidine blue, a supravital stain can accentuate good cytological and nuclear details, enabling three-dimensional view of the cells. It is simple, easily available, cost effective and used for quick reporting.[2] It also permits preservation of cytological material by destaining and re-staining with permanent stains.

This method reduces the turn-around time (TAT). Also with the immediate assessment of adequacy, if the material is found inadequate the procedure can be repeated without any delay. Two major advantages in

this method of staining is that (1) minimal number of staining steps and (2) a review of wet slides without coverslip

Most non-diagnostic results of cytological smears are due to poor sample collection and preparation. Improving the quality of cytological submissions will maximize the likelihood of a meaningful cytological description and a more accurate cytological diagnosis

Aim

The aim of the study is to evaluate the role of toluidine blue.

1. Screening for adequacy of samples and
2. Time efficient and cost-effective method for interpretation of provisional diagnosis
3. Compare it with conventional H&E stain
4. Tissue conservation

MATERIALS AND METHODS

This is a prospective study with a total number of 1200 FNA which were referred to department of cytology over a period of 6 months. Detailed clinical history and all previous lab reports were obtained at the time of FNAC

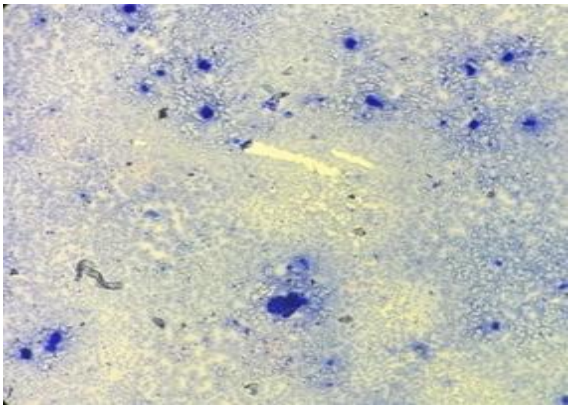

Figure 1: Smears(10X) showing 0.1% toluidine blue for 30 seconds

Following the routine FNA procedure the aspirated material was used to make wet and dry smears and rapid on-site evaluation was done on each smears. The smears were then analyzed to study the efficacy of toluidine blue in adequacy and interpretation

Staining method: Two separate smears were made initially from the aspirated material where one was fixed with absolute alcohol for 10 seconds and other was used as wet film. Smears were then stained with variable concentrations of toluidine blue at different timings and results were documented. [Table 1] depicts the variable concentrations and different timings of toluidine blue.

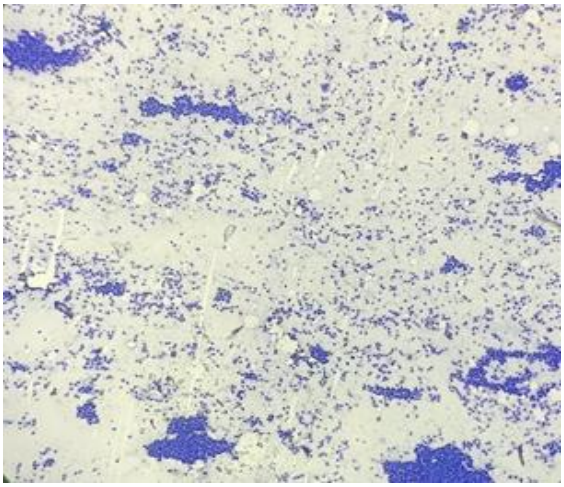

Figure 2: Smears(10X) showing 0.4% toluidine blue for 20 seconds

[Figure 1 and 2] display images of smears stained with toluidine blue at various concentrations and time intervals. In smears stained for 30 seconds with 0.1% toluidine blue, the cellularity is faintly visible, but the cell morphology is not clearly discernible. In contrast, smears stained with 0.4% toluidine blue reveal both cellularity and cell morphology clearly.

Table 1: Comparison of staining method and results yielded:

Stain	Time	Adequacy	Interpretation
0.1% toluidine blue	10 seconds	Cannot be assessed	Cannot be done
0.1% toluidine blue	30 seconds	Can be assessed	Cannot be done
0.4% toluidine blue	10 seconds	Can be assessed	Cannot be properly interpretated
0.4% toluidine blue	20 seconds	Can be assessed	Can be interpretated

Procedure:

1. Fixation in absolute alcohol for 10 seconds
2. Stain with 0.4% toluidine blue for 20 seconds
3. Wash the slide with distilled water
4. View under microscope with/without cover slip

RESULTS

A total of 1200 FNAC of various tissues and organs has been studied with toluidine blue. The distribution of cases based on site and lesions is presented in [Table 2].

Table 2: Distribution of cases according to site.

S. No	Site of FNAC	No. of cases
1.	Lymph node	372
2.	Thyroid	336
3.	Breast	264
4.	Soft tissue	120
5.	Salivary gland	72
6.	Others	36

[Table 3] presents a comparative study of non-neoplastic lesions in various tissues and organs, stained with Toluidine Blue and H&E

Out of 525 non-neoplastic cases, 510 were correctly diagnosed through cytological study using toluidine blue, resulting in an overall accuracy of 98%. Out of 198 cases of reactive lymphadenitis, 9 cases showed discordance with H&E, resulting in an accuracy rate of 95%. Similarly, out of 117 cases of tuberculous lymphadenitis, 110 cases were in concordance with H&E, yielding an overall accuracy rate of 94%.

Table 3: Comparison between non neoplastic lesions.

FNAC site	Non neoplastic	Toluidine blue stain	H&E		Accuracy
			No. of cases concordance	No. of cases discordance	
Lymph node	Reactive lymphadenitis	198	189	9	95%
	Tuberculosis	117	110	7	94%
Breast	Abscess	28	28	-	100%
Soft tissue	Inflammatory	120	120	-	100%
Salivary gland	Inflammatory	26	26	-	100%
Others	Inflammatory	36	36	-	100%
	Total no. of cases	525	510	15	98%

[Figure 3 and 4] is showing microscopy of non-neoplastic salivary gland lesions with both ductal and myoepithelial cells in a myxoid background. Fibroadenoma of breast is showing ductal and epithelial cells arranged in sheets and antler horn clusters [Figure 5 and Figure 6]

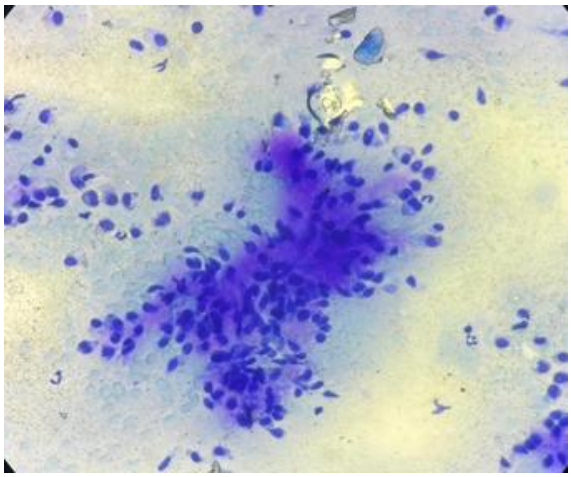

Figure 3: Smears (40X) showing Pleomorphic adenoma

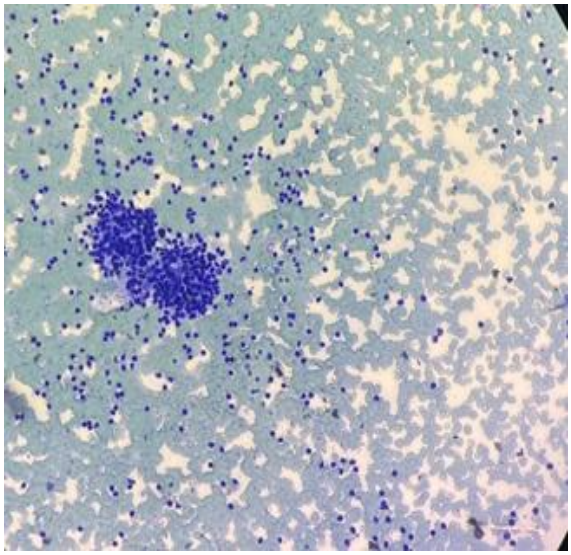

Figure 4: Smears (10X) showing Siladenitis

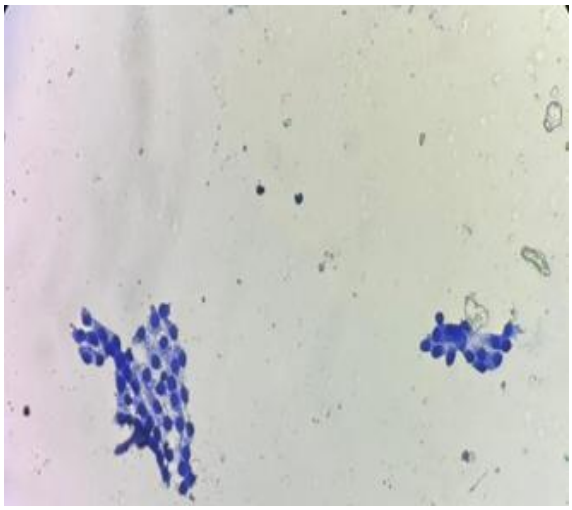

Figure 5: Clusters of ductal epithelial cells(40X)

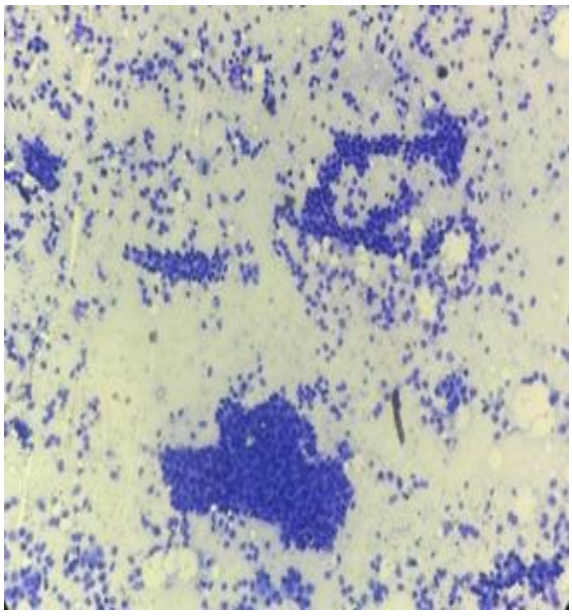

Figure 6: Clusters in fibroadenoma (10X)

[Table 4] illustrates a comparative study of neoplastic lesions in various tissues and organs, stained with Toluidine Blue and H&E in wet smears.

Table 4: Comparison between neoplastic lesions

FNAC site	Neoplastic	Toluidine blue stain	H&E		Accuracy
			No. of cases concordance	No. of cases discordance	
Lymph node	Lympho proliferative	15	9	6	60%
	Secondaries	42	42	-	100%
Thyroid	Benign	290	272	18	93%
	Malignant	46	42	4	91%
Breast	Benign	188	176	12	93%
	Malignant	48	40	8	83%
Salivary gland	Benign	42	39	3	92%
	Malignant	4	2	2	50%
	Total no. of cases	675	622	53	92%

Out of 675 neoplastic cases, 622 were correctly diagnosed, resulting in an overall accuracy rate of 92%.

The accuracy rate for malignant thyroid lesions was 91%, with 42 out of 48 cases diagnosed correctly using Toluidine Blue stain. And in benign there was an accuracy rate of 93%. Some of the lesions of borderline category like follicular neoplasm of thyroid [Figure 7-10] the marked anisonucleosis with some of them showing nuclear pleomorphism can be seen.

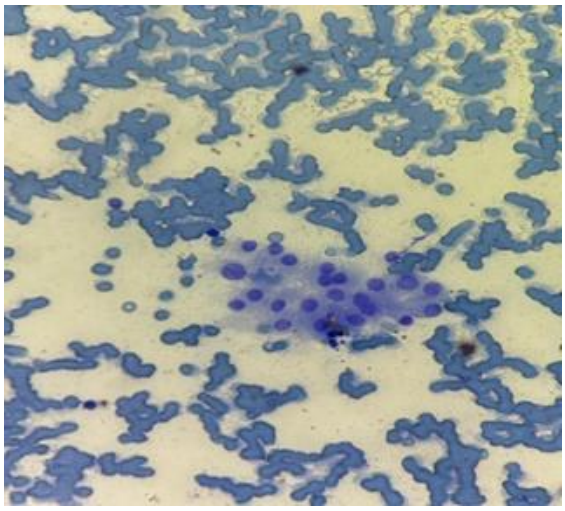

Figure 7: Follicular epithelial cells (40X)

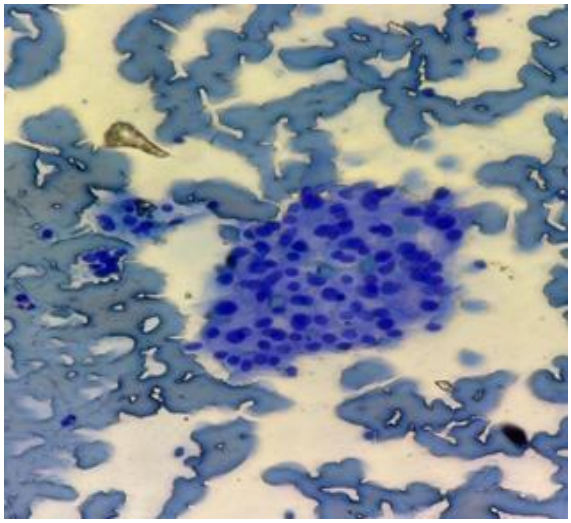

Figure 8: Follicular epithelial cells showing Anisonucleosis (40X)

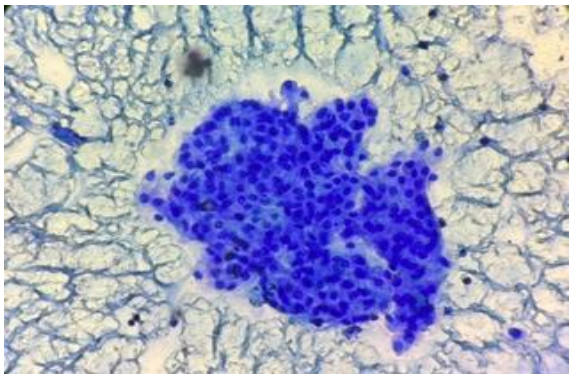

Figure 9: Follicular neoplasm of thyroid (40X)

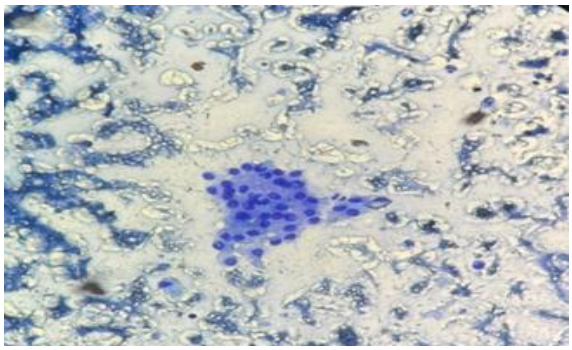

Figure 10: thyroid follicular epithelial cells(40X) showing some nuclear inclusions

[Figure 11 & 12] shows the papillary carcinoma of thyroid with characteristic nuclear features like nuclear grooving, pseudo inclusions and clearing of the nuclei (Orphan annie eye nuclei).

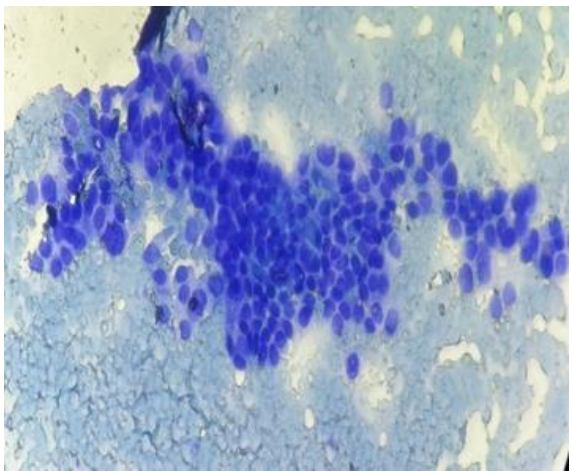

Figure 11: Papillary carcinoma of thyroid(40X)

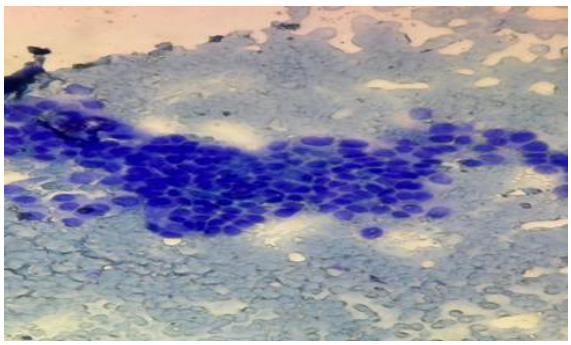

Figure 12: Papillary carcinoma thyroid with nuclear grooving and nuclear inclusions

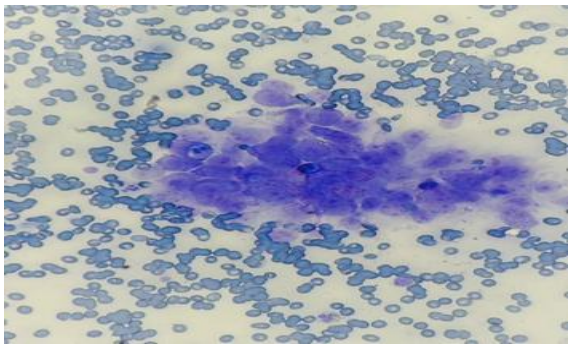

Figure 13: Duct cell carcinoma Breast (40X)

Out of 48 malignant breast lesions, 40 were diagnosed correctly, resulting in an accuracy rate of 83%. [Figure 13 and 14] depict ductal cell carcinoma of the breast, showcasing cells with multiple prominent nucleoli, moderate eosinophilic cytoplasm, and a characteristic comet-tail appearance. The remaining lesions were accurately diagnosed based on cytological examination of wet smears stained with Toluidine Blue

Out of 15 cases of lymphoproliferative disorders, 9 were diagnosed accurately, yielding a diagnostic accuracy rate of 60%. In contrast, all 42 secondary lesions in lymph nodes were correctly identified, demonstrating a 100% concordance with H&E staining.

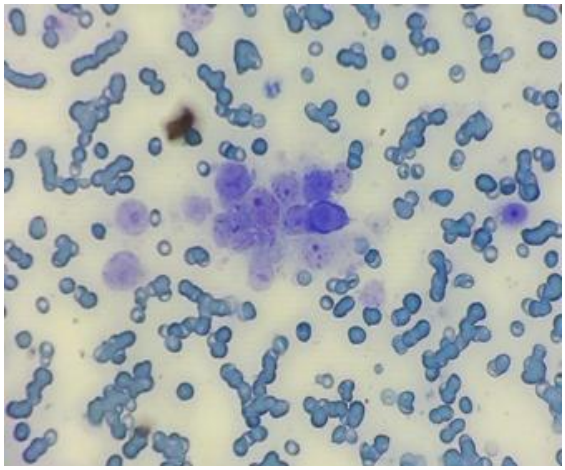

Figure 14: Nuclear features of duct cell carcinoma of breast

[Figures 15 and 16] show metastatic deposits in lymph nodes, characterized by round to polygonal cells with nucleomegaly, moderate eosinophilic cytoplasm, and the presence of tumour giant cells, which are indicative of aggressive malignancies.
[Figures 17 and 18] depict poorly differentiated carcinomatous deposits, suggesting advanced-stage carcinoma with more challenging diagnostic features.
[Table 5] illustrates the overall accuracy for both neoplastic and non-neoplastic lesions

Table 5: Comparison between neoplastic and non-neoplastic lesions

		Toluidine blue	H&E		Accuracy
			No. of cases concordance	No. of cases discordance	
Non neoplastic		525	510	15	98%
Neoplastic	Benign	520	487	33	93%
	Malignant	155	135	20	87%
Total no, of cases		1200	1132	68	94%

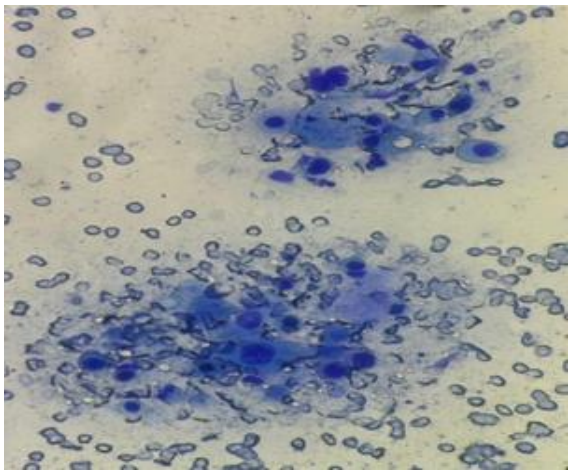

Figure 15: Smears(10X) showing metastatic deposits

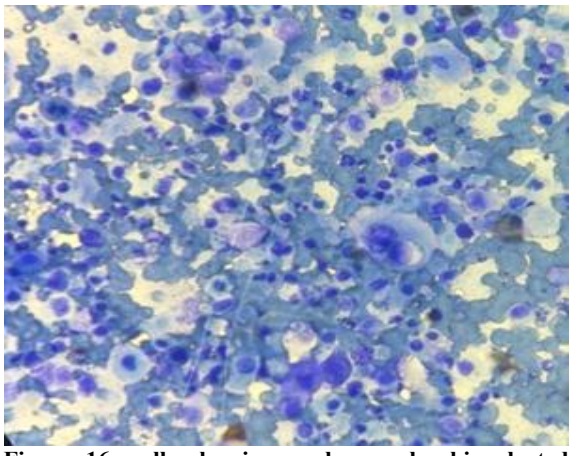

Figure 16: cells showing nucleomegaly; binucleated tumor giant cells (40X)

Out of 1200 cases, 1132 cases (94%) were diagnosed correctly, with a discrepancy of 68 cases between the cytological diagnosis using Toluidine Blue and H&E.

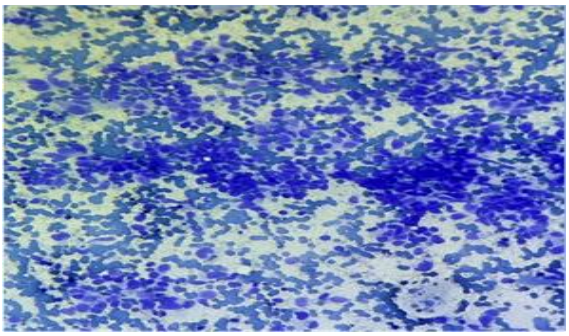

Figure 17: Metastatic deposits in lymph node(10X)

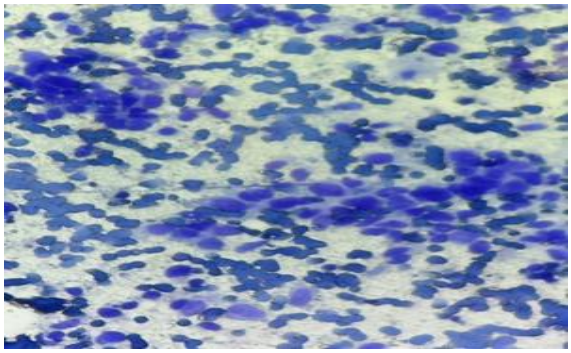

Figure 18: Poorly differentiated carcinomatous deposits (40x)

DISCUSSION

This study aims to examine the cytomorphology of commonly encountered lesions in various tissues and organs, using supravital staining on wet smears. The goal is to identify challenges and limitations in interpretation, as well as to assess the value of providing a rapid diagnosis to the clinician

The success of Fine Needle Aspiration (FNA) depends on five fundamental requirements:

- The sample must be representative of the lesion.
- The sample must have adequate cellularity.
- The smear must be properly prepared.
- The sample must be appropriately processed and stained.
- The reporting and diagnosis must be done by an expert in cytology

In our study we have highlighted the advantages of using supravital staining, such as enhancing cell visibility, improving accuracy in identifying cellular details, and providing real-time diagnostic results. It correlated with the study conducted by Verma et al,[1] where they demonstrated the application of this method in various clinical settings, including oncology, where rapid and accurate diagnosis is crucial for timely treatment planning.

Table 6: Comparison with literature

Article	Focus	Advantages	Challenges
Verma R, Gupta DC:[1]	Supravital staining for rapid cytology diagnosis	Quick, cost-effective, preserves cell morphology	Limited detail, requires expertise
Kothari K, et al.[2]	ROSE for FNAC, rapid on-site sample evaluation	Immediate feedback, reduces repeat procedures	Requires skilled cytopathologist, resource-intensive
Saba K, et al.[3]	Toluidine blue staining vs. Papanicolaou stain for FNAC	Faster than Pap, preserves living cell morphology	Less detailed than Pap stain, requires expertise
Hewer E, Schmitt AM,[4]	Ultrafast toluidine blue staining for ROSE	Quick, cost-effective, enhances ROSE efficiency	Limited detail, requires precise staining technique
Michael CW, et al,[5]	ROSE for thyroid FNAC, benefits and challenges	Immediate diagnostic feedback, improves accuracy	Requires cytopathologist, challenging sample quality
Our study	Toluidine blue as a rapid stain for routine cytological evaluation	Quick interpretation, cost and time effective , better in limited resource setting,	Requires skilled pathologist, selection of smears and accurate staining of smears

Our research on using the supravital stain toluidine blue as a rapid stain is expected to positively impact the implementation of ROSE as well where studies have been conducted emphasizing the implementation of ROSE.[2] So toluidine blue thus helps in giving rapid diagnosis and reducing the turn-around time.

Table 7: Differences between Supravital toluidine blue stain and conventional H &E Stain

Aspect	Supravital Toluidine Blue Staining	H&E Stain
Technique	Stains living cells with toluidine blue to highlight cell structure	Stains fixed cells with multiple dyes to differentiate cell components
Speed	Faster—cells are examined immediately without fixation	Slower—requires fixation and several staining steps
Cell Preservation	Preserves live cell characteristics, offering natural morphology	Cells are fixed, which can alter delicate features
Level of Detail	Less detailed compared to Pap stain, but offers a quicker overview	Provides more detailed cellular and nuclear morphology
Diagnostic Use	Quick, useful for initial diagnosis or screening	Ideal for detailed diagnostic work, especially in cancer detection
Staining Complexity	Simple and cost-effective, requires minimal equipment	More complex and requires more reagents and steps

Ideal Application	Rapid diagnosis in emergency or time-sensitive cases	Comprehensive diagnostic evaluation, especially for cancer
Interpretation Expertise	Requires less training than Pap stain, but still needs expertise	Requires high expertise in cytopathology for accurate results

In our study, we compared toluidine blue with conventional H&E staining, demonstrating its superiority in providing a rapid and accurate diagnosis. Several other studies, such as the one by Saba K et al,[3] have also compared supravital staining with Papanicolaou stain, yielding similar results and further supporting its effectiveness as the preferred stain in many clinical scenarios.

In our study 0.4% of toluidine blue is used which yield accurate results. If inadequate material was aspirated then further aspiration was performed without any delay and thus reducing the time limit and attain adequate cellularity. Speed and simplicity of toluidine blue staining make it a practical solution for enhancing clinical workflows. It enables pathologists to assess the adequacy of samples immediately, thereby reducing the need for repeat aspirations and minimizing patient discomfort. Similar conclusion was drawn up by Hewer et al (4) where they emphasized and demonstrated the diagnostic accuracy of toluidine blue in limited time

As for the staining technique, Ekkehard Hewer et al,[4] used toluidine blue after fixing the samples in absolute alcohol for 5 seconds, while Verma R et al,[1] performed staining on wet films. In our study, smears fixed in absolute alcohol for 10 seconds produced better results.

Table 8: Pitfalls of toluidine blue stain

Pitfalls	Reason
Selection of smears	Some smears may show less cellularity even when taken in single pass So staining of all smears should be done
Over staining	Observed in smears which are largely blood obscured. Time for staining should be reduced by at least 5 seconds in such smears
Under staining	Mainly observed if the stain is not evenly distributed throughout the smear
Uneven staining of cells	Even distribution of stain is to be done
Removal of cover slip to re stain it with H&E	Usually easily removable if done immediately If difficult, freezing method can be done by placing the slide with cover slip facing downwards in 0 to -4 degrees for 10 min and remove gently with microtome blade

Various studies have utilized rapid staining methods such as rapid H&E, ultra- fast PAP, toluidine blue, brilliant cresyl blue (BCB), and Diff-Quik. While Diff-Quik is the most commonly used stain, it is relatively expensive. Toluidine blue, on the other hand, offers several advantages: it is more affordable, and the slides can be destained and reused for PAP or H&E staining. Additionally, toluidine blue provides excellent nuclear detail and allows for easy visualization of three-dimensional structures.

Limitations of the study

- Difficult to appreciate necrosis when compared to conventional H&E stain.
- Borderline lesions in thyroid like Bethesda 3 and 4 can be mis interpretated.
- Highly cellular and blood obscured smears sometimes mimic a malignancy as the stain itself tend to overstain such smears. Nuclear features should be carefully observed.

CONCLUSION

- FNA using toluidine blue as a rapid staining method is a reproducible and reliable way of demonstrating the adequacy and interpretation of material from different tissues and organs..[3]
- Involvement of pathologist in ancillary techniques starts with the management of primary tissue sample. Cytopathology material with adequate cellularity are suitable for molecular studies, immune histochemistry.[4]
- With the use of rapid stain like toluidine blue which is time and cost effective in assessing adequacy and interpretation, tissue conservation of such minimal material without any further procedures paves a new way towards the emerging molecular era.
- Given that cytopathologists are also most closely involved in intraoperative diagnosis, any chance for improvement should be embraced.

REFERENCES

1. Verma R, Gupta DC. Rapid method of cytology diagnosis by supravital staining in FNAC of various tissue and organs. Trop J Path Micro 2018; 4(4):359- 364.doi:10. 17511/ jopm. 2018.i4.11
2. Kothari K, Tummidi S, Agnihotri M, Sathe P, Naik L. This 'Rose' Has no Thorns- Diagnostic Utility of 'Rapid On-Site Evaluation' (ROSE) in Fine Needle Aspiration Cytology. Indian J Surg Oncol. 2019 Dec;10(4):688-698. doi: 10.1007/s13193-019-00981-y. Epub 2019 Sep 12. PMID: 31857767; PMCID: PMC6895318.
3. Saba K, Niazi S, Bukhari MH, Imam SF. Use of supravital toluidine blue staining to improve the efficiency of fine-needle aspiration cytology reporting in comparison to papanicolaou stain. Pak J Med Sci. 2015 Sep-Oct;31(5):1146- 51. doi: 10.12669/pjms.315.8411. PMID: 26649003; PMCID: PMC4641272.

4. Hewer E, Schmitt AM. Ultrafast Toluidine Blue Staining for Rapid On-Site Evaluation of Cytological Smears. Acta Cytol. 2020;64(4):375-377. doi: 10.1159/000505254. Epub 2020 Jan 21. PMID: 31962321.

5. Michael CW, Kameyama K, Kitagawa W, Azar N. Rapid on-site evaluation (ROSE) for fine needle aspiration of thyroid: benefits, challenges and innovative solutions. Gland Surg. 2020 Oct;9(5):1708-1715. doi: 10.21037/gs-2019-catp-23. PMID: 33224848; PMCID: PMC7667097.