IJPRP

Indian Journal of Pathology: Research and Practice

Volume 7 Number 4, April 2018
DOI: http://dx.doi.org/10.21088/ijprp.2278.148X.7418.24

Original Research Article

Diagnostic Accuracy of Rapid Supravital Stainsin Comparison with Conventional Stains in Breast Cytology

Veena Raja[1], Lavanya Rajagopal[2], Chinnaiya Subramaniyam Babu Rajendra Prasad[3]

[1,2]Assistant Professor [3]Professor, Department of Pathology, SRM Medical College and Research Institite, SRM University, Kattankulathur, Chennai, Tamil Nadu 603203, India.

Corresponding Author:

Veena Raja,
Assistant Professor,
Department of Pathology,
SRM Medical College and
Research Institite, SRM
University, Kattankulathur,
Chennai, Tamil Nadu 603203,
India.
E-mail:
drveenaraja88@gmail.com

(Received on 22.02.2018,
Accepted on 31.03.2018)

Abstract

Background: Fine needle aspiration cytology (FNAC) is a very important component of pre-operative investigation in combination with clinical, radiological and other laboratory data. In spite of its advances and advantages, conventional FNAC fails to achieve a 100% accuracy. To improve the accuracy of FNAC, a supravital stained Toluidine blue wet mount (TBWM) preparation of the aspirate and Methylene blue/Eosin (M/E) stained smears are studied

Aim: To assess the diagnostic rapidity of the Toluidine blue wet mount preparation and Methylene blue/Eosin stain in FNAC and to compare the morphological features and results obtained from rapid stains with conventional PAP and H&E techniques in FNAC.

Materials and Methods: The study was conducted on Fine needle aspirates from 96 patients presenting with breast lump in the Department of Pathology. TBWM, M/E stain, H&E stain and Pap stain was done for all 96 cases. Time taken for each stain is assessed and the morphology of rapid stains were compared with the conventional stain. Quality index was calculated for all the stains.

Statistical Analysis: Validation was done using tests like Sensitivity, Specificity, Positive predictive value and ROC curve. The p value ≤ 0.05 was considered significant.

Results: Toluidine blue wet mount takes only 2 minutes for staining followed by Methylene blue/eosin which takes 5 minutes. M/E stain gave the same result as compared to the conventional stains [PAP and H&E]. In TBWM there was difficulty in diagnosing few cases due to three dimensional clusters. The sensitivity and specificity of TBWM were 68% and 94% whereas for M/E stain 99% and 100%. The quality index of PAP stain, H&E stain, M/E stain and TBWM were 0.86, 0.82, 0.72, 0.66 respectively.

Conclusion: TBWM and M/E stain can be routinely undertaken as a supplementary procedure for conventional stains to improve the cellularity and to reduce the time taken for re-sampling.

Keywords: Toludine Blue Wet Mount; Methylene/Eosin; Rapid Cytodiagnosis; Quality Index.

Introduction

Fine needle aspiration cytology (FNAC) technique has become more common and popular nowadays. Though it is not a substitute for conventional histopathology, it should be regarded as a very important component of pre-operative/ pre-treatment investigation in combination with clinical, radiological and other laboratory data [1,2].

Since the majority of lumps are benign, FNAC plays a major role in making a diagnosis and planning appropriate management [3,4].

The diagnostic accuracy of FNAC depends on adequacy and representativeness of sample and good cytomorphological detail without much artifactual distortion. Conventional FNAC has its own advantages and limitations, which are well known to any cytopathologist. In spite of its advances and advantages, conventional FNAC fails to achieve a 100% accuracy. This is partly because of,(i) A lack of sufficient cellularity in desmoplastic lesions.(ii) Wastage of aspirated cells when they stick to the hub and lumen of the needles, (iii) Morphological distortion produced when the cells are trapped in fibrin mesh, (iv) Distortion of fragile cells during smearing, (v) Loss of cell to cell and cell to stromal architecture [1,2].

Hence in an attempt to improve its accuracy, a supravital stained Toluidine blue wet mount (TBWM) preparation of the aspirate and Methylene blue/Eosin (M/E) stained smears are studied which will give additional information regarding the lesion on which FNAC is done. These supravital stains attains good definition of cell outline, cytoplasmic contents and nuclear details.

Therefore this study is aimed at assessing the diagnostic rapidity of the Toluidine blue wet mount preparation and Methylene blue/Eosin stain in FNAC and to compare the morphological features and results obtained from rapid stains with conventional PAP and H&E techniques in FNAC.

Materials and Methods

The study was conducted on Fine needle aspirates from 96 patients presenting with breast lump in the Department of Pathology. Consent was taken from all the patients included in the study. Aspirates yielding very little material was excluded from the study.

Staining Procedure

Toludine Blue Wet Mount Staining

Step 1: A drop of fine needle aspirate is expressed on centre of slide

Step 2: In case of body cavity fluid cytology drop of fluid was placed in the center of slide if fluid was turbid or the fluid was centrifuged at 1500 rpm/min for 10 minutes. The supernatant fluid was discarded. Then a drop of well mixed sediment was placed in the center of slide.

Step 3: Add drop of 0.5% Toluidine blue stain

Step 4: Mix with needle

Step 5: Add a drop of diluted Eosin stain and mix well (optional)

Step 6: Cover with cover slip

Step 7: Wet mount was examined under microscope and cytomorphology was observed.

Alternative Method of Preparation of Wet Mount

This method was tried whenever aspirates were very scanty and adhered to hub of the needle. Under such condition it was very difficult to express the aspirate over the slides.

Step 1: A few drops of toluidine blue stain was aspirated using the same syringe and needle and rinsed.

Step 2: Then the stain mixed material was expressed in the center of slide.

Step 3: A drop of eosin solution was placed next to cell stain mixture and mixed well (optional)

Step 4: Cover with cover slip

Step 5: Wet mount was examined under microscope and cytomorphology was observed.

Methylene Blue Alkaline/Eosin Staining

Step 1: Air dry the smear

Step 2: Fix smear in 95% methanol – 1 min

Step 3: Wash in running tap water

Step 4: 1 Dip in 1% eosin

Step 5: Wash in running tap water

Step 6: 1 dip in Loffler's methylene blue alkaline

Step 7: Wash in running tap water

Step 8: Air dry and mount with DPX

Toludine blue wet mount preparation, Methylene/Eosin stain, H&E stain and Pap stain was done for all 96 cases. Time taken for each stain is assessed and the morphology of rapid stains were compared with the conventional stain.

Assessment of Quality Index

The morphology of the cells was assessed using the following scoring system for all the four stains on all 96 cases using the following scoring system [Table 1].

The maximum score for a single case, taking into account of all the four parameters, was 11. Thus, the maximum possible score in the study was calculated by multiplying the number of cases by 11 for each of four stains. The "Quality index" was obtained by finding out the ratio of actual score obtained to the maximum score possible.

Quality Index = Actual score obtained / maximum score possible [5].

Statistical Analysis

We used the IBM SPSS Software (v.22) to perform the statistical analysis. Validation of rapid cytodiagnostic tests

was done against the gold standard PAP and H&E stains using tests like Sensitivity, Specificity, Positive predictive value and ROC curve. The p value ≤ 0.05 was considered significant.

Results

96 cases with good cellularity where all stains can be performed were chosen for the study. For each case four stains have been done and were analysed by two pathologists for the morphological features and for final diagnosis. Pathologists were blinded for final diagnosis.

Rapidity Assessment

The rapidity of the stains was assessed for all four stains. Toluidine blue wet mount takes only 2 minutes for staining followed by Methylene blue/eosin which takes 5 minutes. [Table 2].

Out of these 96 cases M/E stain gave the same result as compared to the conventional stains [PAP and H&E] except for 1 case of fibroadenoma was diagnosed as phyllodestumor. This is due to high cellularity and mild atypia. In TBWM, difficulty was faced in diagnosing fibrocystic change due to three dimensional clusters and cases showing atypia in fibroadenoma and atypia in Phyllodes was given as suspicious for malignancy in TBWM. Thus the total number of concordant cases by TBWM is 76. [Table 3].

Discordant thyroid lesions by wet mount technique and M/E stain in comparison with conventional stains [PAP and H&E] are shown in Table 4 & 5. The sensitivity, specificity, positive predictive value and negative predictive value of TBWM and M/E stain were 68%, 94%, 75%, 94% and 99%, 100%, 99%, 100% respectively [Table 6].

ROC Curve by comparing rapid stains with conventional stains are shown in figure 1. Area Under Curve for TBWM and M/E were statistically significant with p value <0.05. [Table 7].

The morphology of the cells was assessed using the scoring system for all the four stains on all 96cases and the quality index were calculated [Table 8 & 9]. The salient features of all four stains were shown in Table 10.

Table 1: The scoring system to assess Quality Index

Score	1	2	3
Slide Quality			
Background	Hemorrhage/Necrosis	Clean	
Overall staining	Bad	Moderately good	Good
Cell morphology	Not preserved	Moderately preserved	Well preserved
Nuclear characteristics	Smudgy chromatin	Moderately crisp chromatin	Crisp chromatin

Table 2: Time taken for each staining technique

S. No	Staining Techniques	Time Take for the Procedure
1	Toluidine blue wet mount stain	2 minutes
2	Methylene blue/Eosin stain	5 minutes
3	H&E stain with artificial drying	8 minutes
4	PAP stain with artificial drying	10 minutes

Table 3: Distribution of concordant breast lesions based on stains

Nature of Lesion	Conventional Stains (PAP AND H&E)	Methylene/EOSIN	Wet Mount
Fibroadenoma	23	22	21
Fibrocystic change	19	19	14
Acute inflammatory lesion	6	6	5
Galactocele	5	5	4
Accessory breast tissue	5	5	3
Phyllodes tumor-benign	3	3	2
Keratinous cyst	3	3	3
Fat necrosis	2	2	1
Granulomatous mastitis	1	1	-
Gynecomastia	1	1	1
Suspicious for malignancy	1	1	-
Malignancy	27	27	22
TOTAL	96	95	76

Table 4: Discordant breast lesions by wet mount technique in comparison with conventional stains [PAP and H&E]

Discordant Lesion Number (n=20)	Diagnosis in Conventional Stains [PAP and H&E]	Diagnosis in Wet Mount
1	Fibrocystic change	Acute inflammatory lesion
4	Fibrocystic change	Suspicious for malignancy
1	Fibroadenoma	Suspicious for malignancy
1	Fibroadenoma	Ductal carcinoma
1	Fat necrosis	Acute inflammatory lesion
1	Acute inflammatory lesion	No opinion
1	Granulomatous mastitis	Acute inflammatory lesion
1	Galactocele	No opinion
1	Accessory breast tissue	Ductal carcinoma
1	Accessory breast tissue	Lipoma
1	Phyllodestumor	Suspicious for malignancy
1	Suspicious for malignancy	Benign breast lesion
1	Ductal carcinoma	Fibroadenoma
2	Ductal carcinoma	Suspicious for malignancy
2	Papillary neoplasm	Benign breast lesion

Table 5: Discordant breast lesions by methylene blue/eosin technique in comparison with conventional stains [PAP and H&E]

Discordant Lesion number (n=1)	Diagnosis in Conventional Stains [PAP and H&E]	Diagnosis in Methylene Blue/Eosin
1	Fibroadenoma	Phyllodestumor - Benign

Table 6: Statistical analysis by comparing wet mount with conventional stains in breast lesions

Statistical Parameters	Wet Mount	Confidence Interval	Methylene Blue/Eosin	Confidence Interval
Sensitivity	68 %	70 – 95 %	99%	73 - 100%
Specificity	94 %	92 – 100 %	100%	94 – 100%
Positive predictive value	75 %	84 – 96 %	99%	73 – 100%
Negative predictive value	94 %	84 – 98 %	100 %	94 – 100%

Table 7: Area Under Curve

Test Result Variable(s)	Area Under the Curve				
	Area	Std. Error[a]	p value[b]	Asymptotic 95% Confidence Interval	
				Lower Bound	Upper Bound
WM	0.870	0.045	<0.0001*	0.782	0.957
M/E	1.000	0.000	<0.0001*	1.000	1.000

The test result variable(s): WM has at least one tie between the positive actual state group and the negative actual state group. Statistics may be biased

a. Under the nonparametric assumption

b. Null hypothesis: true area = 0.5

Table 8: The results of estimating the efficacy of four stains (n=100)

Parameter	PAP (n=96)	H&E (n=96)	M/E (n=96)	Wet mount (n=9(
Background				
Hemorrhage/necrosis	8	8	9	10
Clean	88	88	87	86
Overall Staining				
Bad	10	13	20	37
Moderately good	16	27	58	50
Good	70	56	18	9
Cell Morphology				
Not preserved	5	5	10	14
Moderately preserved	30	36	61	70
Well preserved and crisp	61	55	25	12
Nuclear Characteristics				
Smudgy chromatin	13	14	26	32
Moderately crisp chromatin	36	50	55	56
Crisp chromatin	47	31	15	8

Table 9: Overall scores obtained by all four stains with Quality Index

Parameter	PAP	H&E	Methylene Blue/Eosin	Wet mount
Background score	184	184	183	182
Overall staining score	252	235	190	164
Cell morphology score	248	242	207	190
Nuclear characteristics score	226	207	181	168
Actual score obtained	910	868	761	704
Maximum score possible	1056	1056	1056	1056
Quality Index	0.86	0.82	0.72	0.66

Table 10: The salient features of all four stains

Feeatures	PAP	H&E	M/E	TBWM
Smearingtechnique	Important	Important	Important	Nil
Fixation	Immediate fixation	Immediate fixation	Air drying followed by fixation	Nil
Artifacts	Delay in fixation	Delay in fixation	Nil	Air bubbles
Cell loss	Due to wet fixation	Due to wet fixation	Minimal	Minimal
Cell size	Decreased, due to immediate fixation	Decreased, due to immediate fixation	Increased due to air drying	Increased
Cytoplasm	Well appreciated	Well appreciated	Moderately appreciated	Poor
Nucleus	Excellent	Excellent	Excellent	Moderately good
Nucleolus	Distinct	Excellent	Excellent	Excellent, appears immediately
Tissue fragment	Good	Good	Moderate	Moderate
Advantage	Crisp Nuclear details	Very good nuclear details, similar to PAP	Immediate assessment, Good nuclear details	Obtain material from needle hub
Disadvantage	Cell loss due to wet fixation	Cell loss due to wet fixation	Increased nuclear size due to drying	Three dimensional clusters masking the cellular details
Slide preservation	Preserved	Preserved	Preserved	Cannot be preserved
Cost	Expensive	Expensive	Cost effective	Cost effective
Rapidity	10 minutes	8 minutes	5 minutes	2 minutes

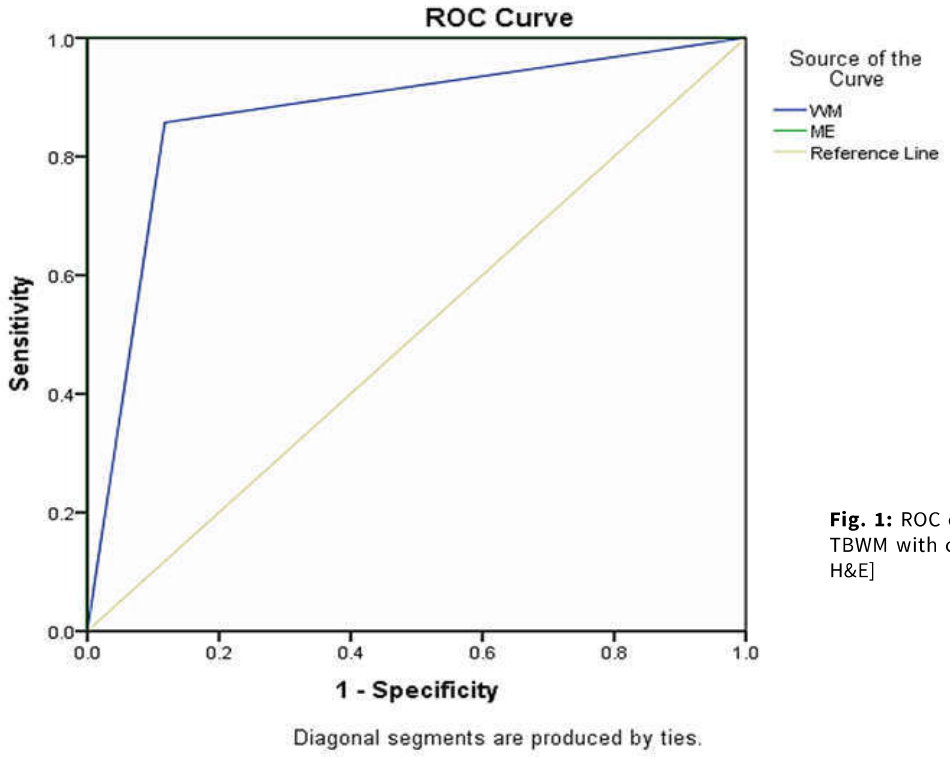

Fig. 1: ROC curve by comparing M/E and TBWM with conventional stains [PAP and H&E]

Table 11: Comparison of diagnostic accuracy of rapid stain with other studies

Authors	Rapid Stain Used	Diagnostic Accuracy
Chandler foot et al.[6]	Neutral red Janus green stain	80%
Silverman et al.[7]	Diff-quik stain	96%
Kusumverma et al.[8]	Rapid MGG stain	97%
Chang et al.[9]	Liu's stain	94.9%
Tsou et al.[11]	Riu's stain	93.5%
Joy MP et al.[3]	Toludine blue stain	98.54%
Sumathy C et al.[2]	Toludine blue wet mount	89%
SumathiS et all.[1]	Toludine blue wet mount	87%
Present study	Toludine blue wet mount	76%
Present study	Methylene blue/eosin	98%

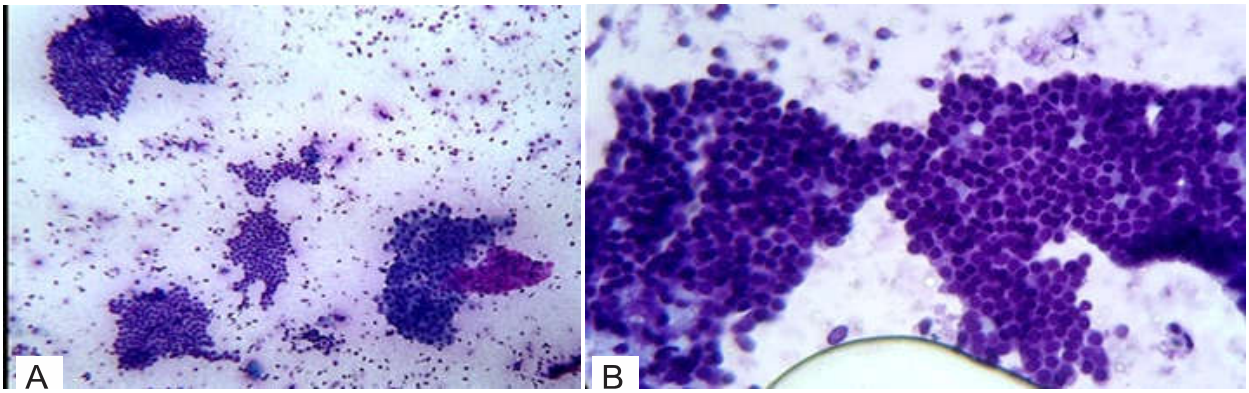

Fig. 2: Fibroadenoma: A – M/E stain (10x), B – TBWM (45x)

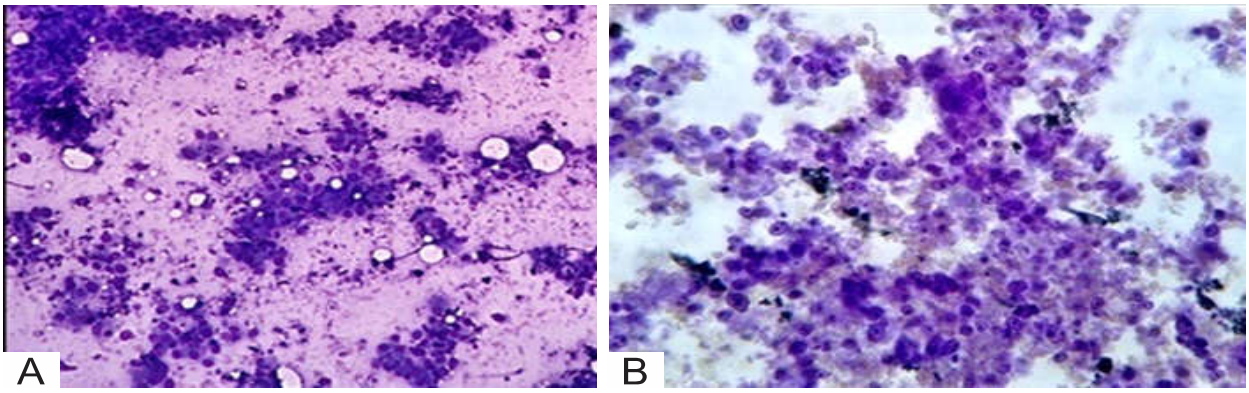

Fig. 3: Ductal carcinoma: A – M/E stain (10x), B – TBWM (45x)

Discussion

FNAC plays a vital role as a rapid diagnostic technique because of its simplicity, cost effectiveness, early availability of results, accuracy and minimal invasion. Chandler foot et. al. (1958), Silverman et. al. (1989) Verma et. al. (1991), Chang et. al. (1993), yang et. al. (1995), Tsou et. al. (1997) experimented various rapid stains such as Neutral red – Janus green, Diff Quik, rapid MGG, Liu's stain, ultra-fast PAP, Riu's stain respectively for immediate diagnosis [6-11]. This work was inspired by the earlier work of Joy, M.P. et. al. in 2003 where they applied toluidine blue as a rapid stain for quick diagnosis of ultrasound guided aspiration cytology [3].

The rapidity of the stains was assessed and found that toluidine blue wet mount stains in 2 minutes followed by methylene blue/eosin in 5 minutes. Rapid staining technique has an advantage of assessing the cellularity and adequacy of the material within few minutes and the re-aspiration can be performed immediately. This will be of much help in USG or CT guided FNAC.

Caya et al in 1984 reported that false negative reports were resulted from unrepresentative aspirates. False negative aspirates may include normal or reactive elements but necrotic material is an additional source of error [12,13]. This problem of sampling error cannot be eliminated entirely in FNAC but it is found reduced by rapid

cytology assessment. This sampling error is reduced in our study by simultaneously doing rapid wet mount study.

Cagle et. al., in 1993 reported that inadequate sampling was solely responsible for 10% false negative report in lung FNAC [14]. In our study the needle and hub are rinsed with toluidine blue stain, which effectively washes all the cells collected in the lumen yielding an improved cellularity.

Degenerated cells and neoplastic cells are more fragile and distorted easily during smearing which created confusion in diagnosis. Trapping of cells within fibrin meshwork also distorted the morphology of cell. Since cytomorphology forms the basis for the cytodiagnosis, artifactual morphological distortion influences the diagnostic accuracy of FNAC [7].

This smearing artifact is avoided in our study since we used wet mount preparations as one of the rapid stain. The disadvantage of this wet mount technique was three dimensional clusters of cells which reduces the diagnostic accuracy in our study.

One of the most important features in cytodiagnosis is the morphology of the nucleus [12]. The advantage of this supravital stain is that the cell structure is well preserved with toluidine blue stain [15]. Supravital stain has a high affinity for DNA and hence absorbed rapidly into the nucleus. As the dysplastic and anaplastic cells contain more nucleic acid, the nuclear stains of tumor cells are very prominent with toluidine blue [16]. Another advantage is the postfixation after air-drying facilitates chromatin staining in M/E stain which will improve the diagnostic accuracy in malignant cases.

Fibroadenoma shows branching sheets of uniform round to oval cells with scanty to moderate cytoplasm, round to oval uniform nuclei with granular chromatin and many bare ovoid nuclei were observed in both M/E and TBWM [Figure 2]. Ductal carcinoma aspirates showed pleomorphic cells with scanty to moderate cytoplasm, large darkly stained nucleus with smudged chromatin and prominent nucleoli. Prominent nucleoli was well appreciated in TBWM than M/E. Sometimes a necrotic material in the background was observed [Figure 3].

In breast lesions the major discrepancy in TBWM was found in diagnosing the benign cases with atypia which was given as suspicious for malignancy. This is due to three dimensional clusters of unfixed cells due to which the morphology was obscured. This was correlated with other studies where toluidine blue was used as wet mount.[2]

The diagnostic accuracy of rapid stains were compared with other studies and shown in Table 11.

The slides of TBWM could not be preserved since the cells were not fixed and should be photographed immediately. This is the major disadvantage of this stain. But it can be preserved for few hours, by sealing the cover slip by applying melted Vaseline or DPX. By this improvement the cytomorphology can be retained for a period of 2 to 3 hours without any morphological distortion and the quick drying of wet mount can also be prevented.

In most of the cases the material gets stuck in needle hub which is very difficult to obtain on the slide. This drawback was overcome by TBWM, where the toluidine blue is directly aspirated on the same needle used for FNAC and the material is expressed on the slide. This technique helps in obtaining the overall material from the needle and will improve the cellularity and helps in supplementing the conventional stains.

M/E stain helps in assessing the cellularity within few minutes which will guide us whether to proceed with the staining or to do re-aspiration. This method helps in reducing the time consumption in cases of USG guided FNAC and in intra operative cytodiagnosis.

Conclusion

Toluidine blue wet mount and Methylene blue/Eosin stain can be used as a rapid diagnostic test. It is also used to assess adequacy of sample especially for deep seated lesions and in USG guided FNAC. It can be used for intra operative cytodiagnosis as an adjunct to frozen section diagnosis. Thus, both stains can be routinely undertaken as a supplementary procedure for conventional stains to improve the cellularity and to reduce the time taken for re-sampling. This work gives a new dimension to the art of FNAC and also opens a new door for further researches in this regard.

References

1. Sumathi S, Mrinalini VR. Supravital stained wet film study of fine needle aspirates: A reliable supplementary diagnostic procedure. Clin Cancer Investig J 2012;1:135-39.
2. Sumathy C, Durai SJ, Swaminathan K, Vallimanalan S, Munavarah SA. Supravital stained rapid wet mount preparation of fine needle aspirates-A cytomorphological study. tejms 2012;3:62-66.
3. Joy MP, Iyer VK, Aron M, Kapila K, Verma K. Rapid staining using toluidine blue: A reliable method for quick diagnosis in ultrasound guided aspiration cytology. Indian J PatholMicrobiol 2003;46:589-92.
4. Erkilic S, Kocer NE. Diagnostic accuracy of toluidine blue stained wet films in effusion cytology. ActaCytol 2006;50:407-9.
5. Idris AAA, Hussain MS. Comparison of the efficacy of three stains used for the detection of cytological changes in Sudanese females with breast lumps. Sudanese J Pub Health 2009;4:275-77.

6. Foot CN, Nelson D, Quist H. Supravital staining of sediments of serous effusions, a simple technique for Rapid Cytological diagnosis. Cancer 1958;11:151-57.
7. Silverman JF, Finley JL, O'Brien KF, Dabbs DJ, Park HK, Larkin EW, et al. Diagnostic accuracy and role of immediate interpretation of fine needle aspiration biopsy specimen from various sites. ActaCytol1989;33:791-96.
8. Verma K, Tiwari MC, Agarwal J, Kapila K. Diagnostic accuracy of immediate interpretation of fine needle aspiration. Indian J Med Res 1991;94:197-99.
9. Chang MC, Chan RD, Ho WL. Intra operative cytology, the use of Liu's stain for immediate diagnosis. Zhonghua Yi XueZaZhi (Taipei) 1993;51:368-75.
10. Yang GC, Alvaraz ll. Ultra fastpapanicolaou stain. An alternative preparation for fine needle aspiration cytology. ActaCytol 1995;39:55-60.
11. Tsou MH, Lin HH, Ko JS. Riu's Stain and the cytologic diagnosis of thyroid fine needle aspiration a single cancer center experience. DiagnCytopathol 1997;16:543-47.
12. Caya JG, Clower LJ, Wollenberg NJ, Tiev TM. Transthoracoc fine needle aspiration cytology, Analysis of 82 patients with detailed verification criteria and evaluation of false negative cases. Am J ClinPathol 1984;82:100-03.
13. Winning AJ, Seed MJ, Husain WA, Metaxas OAN. Interpretation of Negative results in fine needle aspiration of discrete pulmonary lesions. Thorax 1986;41:875-9.
14. Cagle PT, Kovach M. Ramzy I. Causes of false results in transthoracic fine needle aspirates. ActaCytol 1993;37: 16-20.
15. David J, Henry CK, Feider, Splittgerber GF. Toluidine blue dye as a breast localization marker. Am J Roentqenol 1989; 153:261-63.
16. Dudgeon LS, Patrick CV. A new method for the rapid microscopical diagnosis of tumors. Br J Surg 1927;15:250-61.