Original Article

Supravital-stained wet film study of fine needle aspirates: A reliable supplementary diagnostic procedure

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ABSTRACT

Background: Fine needle aspiration cytology (FNAC) is a simple, rapid, reliable, and cost-effective method in diagnosing mass lesions. In spite of its advances and advantages, conventional Hematoxyline and Eosin (H and E)-stained wet-fixed smear of FNAC fails to achieve 100% accuracy. To improve the accuracy of cytodiagnosis, toludine blue (TB)-stained wet film preparation of fine needle aspirates is supplemented along with conventional wet-fixed smear. We have assessed the morphology and accuracy of supravital-stained (TB) wet film study of FNAC, which has not been previously reported. **Materials and Methods:** A total of 197 fine needle aspirates from various body sites were studied both in supravital toludine blue (TB)-stained wet film and hematoxylin and eosin (H and E)-stained wet-fixed smear preparation. The results were interpreted with final diagnosis made by histopathological study, clinical, radiological follow-up and were statistically analyzed. **Results:** For the entire series, TB-stained wet film study gave a sensitivity of 93.7%, a specificity of 98%, a positive predictive value (PPV) of 96.6%, a negative predictive value (NPV) of 96.9%, and an efficacy of 96.3%. H and E-stained wet smear study revealed a sensitivity of 86.2%, specificity of 97.9%, PPV of 95.4%, NPV of 93.4%, and an efficacy of 93.2%. The combined wet film and wet smear study results showed a sensitivity of 98%, specificity of 99.2%, PPV of 98.4%, NPV of 98.9%, and an efficacy of 98.6%. The decreased sensitivity of wet smear study due to inadequate cellularity, loss of cell sample during fixation and staining, artifactual morphological distortion were minimized by supplementary wet film study and that yielded high accuracy rate. **Conclusion:** Wet film study gave a good cytomorphological picture and this immediate interpretation was useful for assessing the adequacy of material. False negative and false positive reports were reduced significantly when we combined this toludine blue-stained wet film study and wet smear study. Therefore, it could be regularly undertaken as a supplementary diagnostic procedure for wet smear to improve the diagnostic accuracy.

Key words: Fine needle aspiration cytology accuracy, rapid stain, supravital stain, toludine blue, wet film

INTRODUCTION

Fine needle aspiration cytology (FNAC) is a cost-effective, low risk, accurate tool for diagnosing disease in many organs. Various studies had been done to reduce pitfalls in cytodiagnosis and to improve the diagnostic accuracy. The diagnostic accuracy of FNAC depends on adequacy of sample, representativeness of the sample, and good cytomorphological detail without much artifactual distortion. Several authors studied about the immediate cytological evaluation using rapid stains to assess the sample adequacy and to improve the diagnostic accuracy.[1-3] Various stains had been used for this rapid staining technique.[4-10] We preferred toludine blue stain as a rapid stain for wet film study. Many authors applied this rapid staining technique for either air-dried or wet-fixed smear preparation, whereas only few studies were focused on the application of rapid stains in wet film of effusion fluid.[11,12] We had examined supravital toludine blue (TB) stain for wet film study of cytological materials obtained from various sites to minimize morphological distortion by smearing and to improve the diagnostic accuracy. In this paper, cytomorphology of wet film study of cytological materials using supravital (TB) stain and comparison of diagnostic accuracy of wet film study, Hematoxyline and Eosin (H and E)-stained wet smear study alone and combined with wet film study were presented.

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MATERIALS AND METHODS

The study materials include fine needle aspirates, obtained from various sites in 197 patients.

Stains

1 Supravital stain - 0.5% aqueous solution of toludine blue
2. Hematoxyline and Eosin (H and E) stain

Study procedure

Fine needle aspiration was performed using 21-23 G needles attached to 5-10 ml syringes. Following the needle placement, the aspirate was obtained by agitating the needle tip within the lesion. Then, the aspirates were expressed over the slide, and smear was made. Immediately, slides were put in fixative and stained with Hand E stain. (Wet-fixed smear). For wet film study, aspirated material was expressed over slide, and a drop of toludine blue stain was kept aside. Materials were mixed with stain and covered by cover slip, and the margins were sealed with DPX.

Suppose the materials were scanty and adhered to the hub of needle, the needle was rinsed with toludine blue stain. Then, it was expressed over slide, covered with cover slip, and sealed. Now the adequacy, morphology of wet film preparations were interpreted, documented in the cytology requisition form and compared with wet smear diagnosis. The results of wet film and wet smear interpretation were compared with final diagnosis made by histopathological study, clinical and radiological follow-up.

RESULTS

Cytological materials were obtained from various sites like lymph node, thyroid, breast, soft tissue, body cavity fluids, salivary gland, and bone and deep-seated mass lesions under CT guidance from 197 patients. Sample adequacy, cytoplasm, and nuclear details were appreciated in both wet film (TB) and wet smear (H and E) preparation, and diagnosis was made. Cytological diagnoses were categorized as malignant, benign, inflammatory, and unsatisfactory. No further attempt was made to type the tumor and states the nature of inflammatory lesion. Unsatisfactory category included inadequate cellularity and poor cytomorphology by necrosis, hemorrhage, and artifact. Then, it was statistically analyzed with final diagnosis (FD). Table 1a - We had 62 malignant cases on FD, of which 59 were called as malignant, 2 as benign and 1 as inflammatory in wet film study. Wet smear study detected only 50 cases as malignant, 2 as benign, 3 as inflammatory, and 7 as unsatisfactory for diagnosis. But, combined wet film and wet smear study detected 60 malignant cases with only 2 false negative reports. The sensitivity for diagnosing malignant condition was 95.2%, and 80.6% for wet film and wet smear study, respectively. It was increased to 96.7% by our combined wet film and wet-fixed smear study. Of the 78 benign cases on FD [Table 1b], 74 were reported as benign, 1 as inflammatory and 3 as unsatisfactory for diagnosis in wet film study, whereas wet smear study detected 69 benign cases and 2 as inflammatory, 7 as unsatisfactory reports. However, combined study correctly detected 76 benign cases and 1 as inflammatory and 1 as unsatisfactory. The sensitivity for diagnosing benign condition was increased to 97.4% by combined study, whereas it was 94.9% for wet film and 88.5% for wet smear study. We had 57 inflammatory cases, of which wet film study [Table 1c] detected 52 cases as inflammatory, 3 as benign, and 2 as unsatisfactory reports. Wet smear study reported 51 cases as inflammatory, 1 as malignant, and 5 as unsatisfactory reports. However, combined study detected all 57 inflammatory cases and no false negative reports. Therefore, we have got 100% sensitivity for diagnosing inflammatory condition by combined study, whereas it was 91.2% and 89.5% for wet film and wet smear study, respectively.

Table 1a: Correlation of 62 malignant cases on final diagnosis with wet film (TB stain), wet smear (H and E stain), and combined study diagnosis at different sites

Body site	Total No. of Aspirates	Wet film (TB stain)				Wet smear (H and E Stain)				Combined wet film (TB stain) and wet smear (H and E Stain) diagnosisflammatory (57)			
		M	B	Inflam	Unsat	M	B	Inflam	Unsat	M	B	Inflam	Unsat
Lymphnode	62	20	-	1	-	18	-	2	1	20	-	1	-
Thyroid	49	4	-	-	-	4	-	-	-	4	-	-	-
Breast	41	16	1	-	-	15	2	-	-	17	-	-	-
Soft tissue	14	3	-	-	-	2	-	-	1	3	-	-	-
Body fluids	14	6	-	-	-	4	-	1	1	6	-	-	-
Salivary gland	7	1	1	-	-	1	-	-	1	1	1	-	-
Bone	5	4	-	-	-	3	-	-	1	4	-	-	-
Deep viscera	5	5	-	-	-	3	-	-	2	5	-	-	-
Total	197	59(TP)	3(FN)			50(TP)	12(FN)			60(TP)	2(FN)		
Sensitivity			95.2%				80.6%				96.7%		

M: Malignant, B: Benign, Inflam: Inflammatory, Unsat: Unsatisfactory, TP: True positive, FN: False negative

Table 1b: Correlation of 78 benign cases on final diagnosis with wet film (TB stain), wet smear (H and E stain), and combined study diagnosis at different sites

Body site	Total No. of Aspirates	wet film (TB stain)				wet smear (H and E Stain)				Combined wet film (TB stain) and wet smear (H and E Stain) mmatory (57)			
		B	M	Inflam	Unsat	B	M	Inflam	Unsat	B	M	Inflam	Unsat
Lymphnode	62	-	-	-	-	-	-	-	-	-	-	-	-
Thyroid	49	37	-	1	1	35	-	2	2	37	-	1	1
Breast	41	20	-	-	2	19	-	-	3	22	-	-	-
Soft tissue	14	11	-	-	-	9	-	-	2	11	-	-	-
Body fluids	14	-	-	-	-	-	-	-	-	-	-	-	-
Salivary gland	7	5	-	-	-	5	-	-	-	5	-	-	-
Bone	5	1	-	-	-	1	-	-	-	1	-	-	-
Deep viscera	5	-	-	-	-	-	-	-	-	-	-	-	-
Total	197	74(TP)		4(FN)		69(TP)		9(FN)		76(TP)		2(FN)	
Sensitivity		94.9%				88.5%				97.4%			

M: Malignant, B: Benign, Inflam: Inflammatory, Unsat: Unsatisfactory, TP: True positive, FN: False negative

Table 1c: Correlation of 57 inflammatory cases on final diagnosis with wet film (TB stain), wet smear (H and E stain), and combined study diagnosis at different sites

Body site	Total No. of Aspirates	wet film (TB stain)				wet smear (H and E Stain)				Combined wet film (TB stain) and wet smear (H and E Stain) mmatory (57)			
		Inflam	M	B	Unsat	Inflam	M	B	Unsat	Inflam	M	B	Unsat
Lymphnode	62	40	—	-	1	37	1	-	3	41	-	-	-
Thyroid	49	2	-	3	1	6	-	-	-	6	-	-	-
Breast	41	2	-	-	-	2	-	-	-	2	-	-	-
Soft tissue	14	-	-	-	-	-	-	-	-	-	-	-	-
Body fluids	14	8	-	-	-	6	-	-	2	8	-	-	-
Salivary gland	7	-	-	-	-	-	-	-	-	-	-	-	-
Bone	5	-	-	-	-	-	-	-	-	-	-	-	-
Deep viscera	5	-	-	-	-	-	-	-	-	-	-	-	-
Total	197	52(TP)		5(FN)		51(TP)		6(FN)		57(TP)		0(FN)	
Sensitivity		91.2%				89.5%				100%			

M: Malignant, B: Benign, Inflam: Inflammatory, Unsat: Unsatisfactory, TP: True positive, FN: False negative

The study of morphology of individual cell was on great focus in our study since individual cell morphology varied in wet smear and wet film preparation. Cytomorphology was well-appreciated in wet film study since it showed 3 dimensional views of unfixed cells. Nuclear features of malignancy, especially hyperchromatism, anisokaryosis, nucleoli, and nuclear membrane irregularity, were well seen in our toludine blue-stained wet film cyto preparations [Figures 1-4]. In our study, papillary carcinoma of thyroid showed papillary sheets of follicular cells and nucleus showed small prominent basophilic nucleoli predominantly than nuclear grooving, and some showed pale nuclear inclusion. However, we found that tumor cell with definite cytoplasmic criteria for diagnosis did not have this similar feature in toludine blue staining. This problem was noted in our study, particularly in identifying Hurthle cells where the cytoplasmic granules could not be identified giving a 3 false negative reports [Table 1c] for Hashimotos thyroiditis. Moreover, here cells were examined in fresh state, so all the cells appeared larger than those in H and E-fixed smear. Hence, the lymphocytes were falsely interpreted as naked follicular epithelial cells.

One problem in this wet mount study was we could not preserve the slides since the cells were not fixed. In cases where slides have to be preserved for few hours, the cover glass over the sample was sealed off by applying melted Vaseline or DPX. This sealing helped to retain cytomorphology for a period of 2 to 3 hours without morphological distortion and it also prevented quick drying of wet mount.

DISCUSSION

Our study was aimed at improving the diagnostic accuracy of H and E-stained wet-fixed smear study with additional information from toludine blue-stained wet film study. Many studies had been focused on FNAC to improve the diagnostic accuracy by minimizing false negative reports. One reason for false negative reports in FNAC was due to unrepresentative sample.[13] This problem of sampling error could not be eliminated entirely in FNAC, but it was found reduced by this rapid wet film cytology assessment. One study of FNAC lung showed that inadequate sampling was solely responsible for 10% false negative report.[14] The decreased sensitivity of wet smear evaluation of our study was mostly due to this problem. It was minimized by our supplementary wet film study where we had

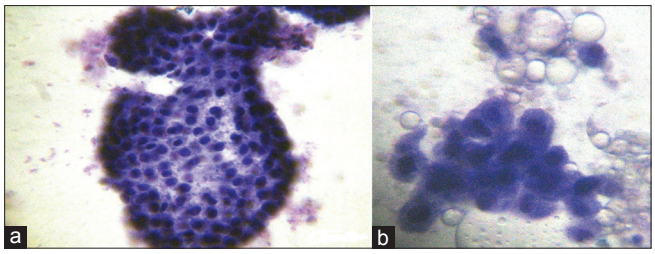

Figure 1: (a) Fibro adenoma breast - Photomicrograph shows papillary sheet of monomorphic duct epithelial cells with bare nuclei in the background (b) Carcinoma breast - Photomicrograph shows cluster of pleomorphic duct epithelial cells having hyper chromatic anisokaryotic nuclei with scattered single cells

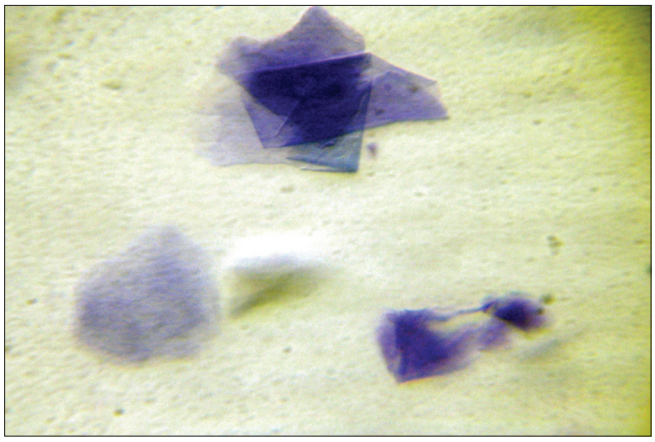

Figure 3: Epidermal cyst - Photomicrograph shows scattered polyhedral anucleated squamous cells

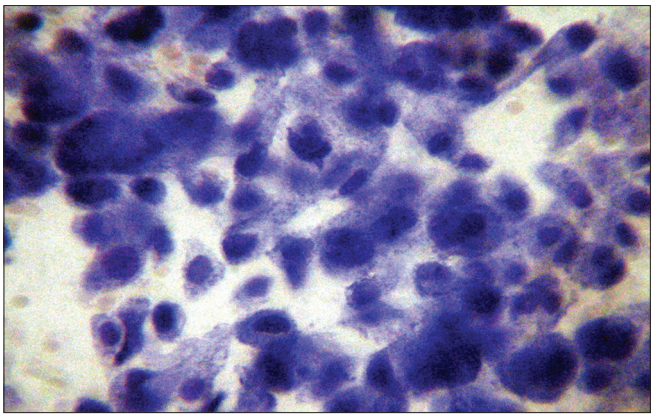

Figure 2: Anaplastic carcinoma of Thyroid - Photomicrograph shows dyscohesive highly pleomorphic cells with hyper chromatic and anisokaryotic nuclei

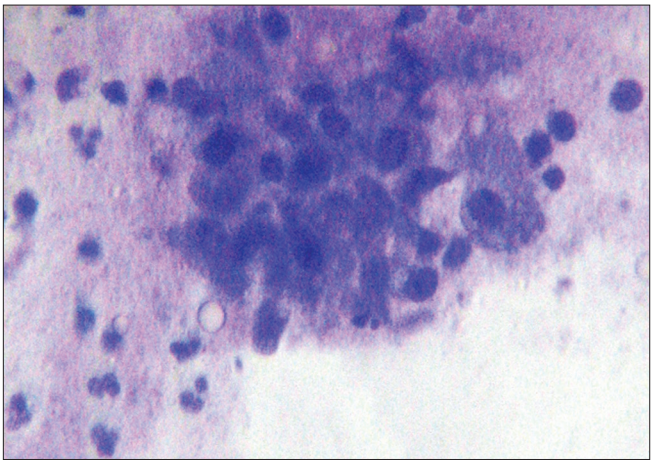

Figure 4: Tuberculous Lymphadenitis - Photomicrograph shows cluster of elongated epithelioid cells with vesicular nucleus, prominent nucleoli, and histiocytes in scattered lymphocytic background

assessed the cellularity immediately. In wet film study, the needle and hub were rinsed with toludine blue stain, which effectively washed all the cells collected in the needle hub and 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. We had noticed this problem in our wet smear study, especially for diagnosing deep-seated mass lesions. This artifact was minimized in our wet film study. Moreover, this wet film study also gave an additional advantage of appreciating cells in three-dimensional view, and cytomorphology was well appreciated. Loss of cell sample during fixation in alcohol and subsequent staining process also pose a major problem in arriving inconclusive diagnosis.[15] It was especially true in case of sampling of cyst and body fluids. It was avoided in our study by doing wet film cytological examination. Here, cells were examined without fixation by supravital staining. Therefore, there was no loss of cell sample and gave sufficient cellularity to render a rapid diagnosis.

One of the most important features in cytodiagnosis was the morphology of the nucleus. In our study, we had found excellent nuclear detail provided by toludine blue stain, enabling an accurate diagnosis. The nuclear features of malignancy were well appreciated in our wet film TB-stained study. This hyper chromatic nature of malignant nucleus was due to increased toludine blue dye uptake as a result of increased DNA, RNA content of the malignant nucleus. In addition, malignant epithelium contains intracellular canals that are wider than normal epithelium. This is the factor that would enhance penetration of the dye.[16] The diagnosis of malignancy was confirmed in all our patients by pathological, clinical, and radiological follow-up. It was important to note that no benign case was reported as malignant in our study. But, we had missed one salivary gland malignancy by both wet film and wet smear study, which was reported as well-differentiated mucoepidermoid carcinoma on FD. However, false negative reports were reduced from 12 to 3 for diagnosing malignant condition by doing supplementary rapid stain study. The results of our study of rapid cytodiagnosis are comparable with those of the earlier works done by many authors.[1,3-12]

Table 2: Correlation of statistical analysis

Statistical test	Wet film (TB stain) %	Wet smear (H and E stain) %	Combined wet film and wet smear %
Sensitivity	93.7	86.2	98
Specificity	98.1	97.9	99.2
Positive predictive value	96.6	95.4	98.4
Negative predictive value	96.9	93.4	98.9
Percent false positive	1.85	2	0.73
Percent false negative	6.2	13.6	1.9
Efficacy	96.3	93.2	98.6

Statistical analysis of entire series of our study [Table 2] showed the sensitivity, specificity, positive predictive value, negative predictive value, and efficacy of 93.7%, 98.1%, 96.6%, 96.9%, and 96.3% for wet film study and 86.2%, 97.9%, 95.4%, 93.4%, and 93.2%, respectively, for wet smear study. However, combined study gave a sensitivity of 98%, specificity 99.2%, positive predictive value 98.4%, negative predictive value 98.9%, and an efficacy of 98.6%. False positive rate was well decreased to 0.73%, and false negative rate was 1.9% by our combined study. The decreased sensitivity of H and E alone due to inadequate cellularity, loss of cell sample during fixation and staining, and artifactual morphological distortion was overcome by supplementary wet mount study and that yielded high accuracy rate.

Our wet film study had some limitations. Since TB stain is a good nuclear stain, we found difficulty in identifying cells with definite cytoplasmic features like hurthle cells. It was the reason for the decreased sensitivity in diagnosing inflammatory thyroid pathology by wet film study. However, this sensitivity was increased by our combined H and E-stained wet smear study. Another problem in this wet film study was that we could not preserve the slides permanently. In order to overcome this limitation, wet film study could be done as a supplementary diagnostic procedure rather than a substitute for wet-fixed smear study.

This study concluded that the supravital-stained wet mount FNAC was useful as a simple, reliable, and cost-effective rapid staining method. It helped to obtain sufficient cellularity in less cellular fibrotic lesions. It was also used to assess adequacy of sample, especially for deep-seated lesions and to minimize false negative results. The cytomorphology was well appreciated in wet film study and it improved the diagnostic accuracy of conventional H and E-stained wet-fixed smear study. So, it could be routinely undertaken as a supplementary procedure for wet smear study.

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