

Comparative Study of Modified Ultrafast Papanicolaou Stain with Routine Papanicolaou, Haematoxylin & Eosin and Giemsa Stain in Cytology.

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Abstract

- Background:** Diagnostic cytology is the science of interpretation of cells that are either exfoliated from epithelial surfaces or removed from various tissues. Good screening makes the accurate diagnosis with minimum efforts. Need for minimal turnaround time have led to various innovations in staining techniques that require lesser staining time with unequivocal cell morphology.
- Aim:** To compare the use of the Modified Ultra-fast Papanicolaou (MUFP) stain with the routine Papanicolaou (Pap), Haematoxylin–Eosin (H&E), and Giemsa stain.
- Materials and Methods:** This was a cross-sectional observational study carried in a tertiary care hospital over a period of one year. Samples from 80 patients comprising 50 FNAC and 30 body fluids were included in the study. The subjects were evaluated clinically, and history taken according to the predesigned proforma. After taking the consent and preparing the patient for aspiration, FNAC was performed taking full aseptic precautions. Data was then statistically analysed.
- Results:** Cases ranged from 18-93 years of age. Male to female ratio of 0.48:1. Out of 80 cases, 50 cases were fine needle aspirations (FNA) and 30 body fluids. Out of the 80 samples, breast FNA comprised 20, followed by ascitic fluid 16, thyroid FNA 16, pleural fluid 14, lymph node FNA 12 and salivary gland FNA 2 cases. MUFP had significantly high mean scores in comparison to other stains for all FNA and body fluid smears with better cytomorphological features.
- Conclusion:** MUFP staining is easily applicable to FNA and body fluids and provides superior cytomorphological picture in comparison to Pap, H&E and Giemsa stains. MUFP stain is rapid and reliable than other stains in benign as well as malignant lesions and is extremely useful in developing countries like India.

Keywords: Modified Ultra-fast Papanicolaou, Cytological Stains, Giemsa stain, Pap, H&E

Introduction

In exfoliative cytology, cells are collected after they have been either spontaneously shed by the body e.g. in pleural and peritoneal effusions or manually scraped/brushed off of a surface. Mechanical exfoliation includes Pap smears or bronchial brushings and lavage.

Fine needle aspiration cytology (FNAC) is simple, safe, quick, reliable and inexpensive technique used to obtain

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cells, tissues through a thin needle (22-27G) for the purpose of diagnosis and management of masses. It is regarded as an important component of pre-operative/pre-treatment investigation in combination with clinical, radiological and other laboratory data.

The value of FNAC is not only limited to neoplastic conditions but is also used in diagnosing non-neoplastic conditions like inflammatory, infectious, degenerative and congenital conditions in which samples can be used for microbiological and biochemical analysis [1].

Staining is an auxiliary technique used in microscopy to enhance contrast in the microscopic image. Commonly used stains for cytological purposes are Pap, H&E and May-Grunwald Giemsa (MGG). Pap stain advocated by

Papanicolaou in 1942 is the best stain for nuclear chromatin with fixation and staining procedure requiring 20-40 minutes, hence it is the preferred stain for FNAC [2]. Need for minimal turnaround time have led to various innovations in staining techniques that require lesser staining time with unequivocal cell morphology.

Ultrafast Pap stain enhances the quality of the stain and reduces the procedure time to 90 seconds with the chief limitation being availability of some reagents, hence Modified Ultrafast Papanicolaou (Modified UFP) stain was developed by Khodke *et al.* and Kamal *et al.* [3,4] MUFP uses locally available reagents *i.e.* Gill's/Harris Haematoxylin omitting Orange-G. The total time required in MUFP staining is 130 seconds.

The present study was undertaken to assess the use of MUFP stain in FNAC of various organs and body fluids and to compare its efficacy with conventional stains and to study its applicability in routine staining.

Aims and Objectives

To compare the utility of Modified Ultra-fast Papanicolaou stain with the routine Pap, H&E, and Giemsa stain

Material and Methods

This cross-sectional observational study was carried out in the Department of Pathology over a period of one year *i.e.* from November 2018 to October 2019 after approval by Institutional Ethics Committee.

Samples from 80 patients comprising 50 FNAC and 30 body fluids were included in the study. For FNAC, the patients were evaluated clinically, and the history taken according to the predesigned proforma. After taking the consent and preparing the patient for aspiration, FNAC was performed taking full aseptic precautions.

For body fluids, samples received in the cytology section were grossly examined for colour and volume and then centrifuged at 3000 rpm for 10 minutes, the supernatant was discarded, and thin smears were made from the sediment.

For preparation of the smear, the aspirated material from FNAC and the sediment from the body fluids were spread on the glass slides and 4 smears were prepared for each sample. These were stained with H&E, Pap, MGG, and Modified UFP stains.

For H&E and Pap staining, smears were fixed in 95% isopropyl alcohol or ethyl alcohol and ether in equal parts. For MGG staining, smears were air dried. Also smears for Modified UFP staining were air dried and processed within 30 minutes of preparation.

MUFP Staining Procedure

- Air dried smears were rehydrated with normal saline for 30 seconds.
- Fixed in Alcoholic Formalin for 10 seconds.
- Dipped in water – (6 slow dips).
- Stained with Gill's Haematoxylin for 30 seconds.
- Dipped in tap water – (6 slow dips).
- Dehydrated in 95% isopropyl alcohol – (6 dips).
- Stained with EA-36 for 15 seconds.
- Dehydrated in 95% isopropyl alcohol – (6 dips).
- Dehydrated in 100% isopropyl alcohol – (6 dips).
- Cleared in Xylene – (10 slow dips).
- Mounted in Dibutylphthalate polystyrene xylene (DPX).

Total time required for staining was: 130 seconds.

Smears were prepared from FNA of various organs such as breast, thyroid, lymph node and salivary gland. Similarly, smears were also made from body fluids *viz.* ascitic and pleural fluid. Smears were then examined under light microscope.

Staining Quality Analysis

On these smears, using the scoring system for the assessment of staining quality as described by Shinde and Pandit (5), quality of each of the four stains was assessed as follows:

Parameter	Scores		
	1	2	3
1. Background	Haemorrhagic	Clear	-
2. Overall staining	Bad	Moderate	Good
3. Cell morphology	Not preserved	Moderately preserved	Well preserved
4. Nuclear characteristics	Smudgy chromatin	Moderately crisp chromatin	Crisp chromatin

On the basis of above four parameters, the maximum score for a single case was 11. The maximum possible score in the study was calculated by multiplying the number of cases by 11 for each of the four stains.

- A "Quality Index" was obtained for each of the four stains.
- Quality Index = Actual score obtained/Maximum score possible.
- Then, the Quality Index for each of the four stains was compared.

The data was analysed using computer software Microsoft Excel and SPSS version 21.0 for Windows. All the values were interpreted statistically using "Analysis of Variance" (ANOVA) and "Post-hoc Bonferroni t-test". All the results were analysed considering statistical significance at a level of $p < 0.05$.

Observations and Analysis

Patients in the study ranged in 18-93 years with maximum number in the age group of 25-44 years (40%). Females outnumbered males in the study comprising of (67.50%) cases with M:F ratio of 0.48:1. Out of 80 cases, 50 cases (62.5%) were fine needle aspirations (FNA) and 30 cases (37.5%) were body fluids.

Out of the total 80 cases sampled, breast FNA comprised 20 (25%) cases, followed by ascitic fluid 16 (20%), thyroid FNA 16 (20%), pleural fluid 14 (17.5%), lymph node FNA 12 (15%) and salivary gland FNA 2 (2.5%) cases.

Table 1: Distribution of Cases according to Age, Gender, Type of Sample and Organ

Characteristic	No. of cases n (%) (N=80)
Age group (in years)	
<24	9 (11.25)
25-34	19 (23.75)
35-44	13 (16.25)
45-54	9 (11.25)
55-64	9 (11.25)
65-74	12 (15)
≥75	9 (11.25)
Gender	
Male	26 (32.5)
Female	54 (67.5)
Type of Cytological sample	
FNAC	50 (62.5)
Body Fluid	30 (37.5)
Organ / Site	
Breast	20 (25)
Lymph node	12 (15)
Salivary gland	2 (2.5)
Thyroid	16 (20)
Ascitic fluid	16 (20)
Pleural fluid	14 (17.5)

Out of the total 20 breast lesions, 15 (75%) cases were benign in nature and 5 (25%) cases were diagnosed as malignant. Fibroadenoma was the most common diagnosis among the benign lesions on breast FNA (73.33%). On FNA smears of fibroadenoma, the bipolar naked nuclei were poorly stained by MUF. Cytological diagnosis in lymph node aspirates was benign in 8 (66.67%) cases and malignant in 4 (33.33%) cases. In salivary gland aspirates cytological diagnosis of pleomorphic adenoma of parotid gland was made in 2 cases. Nodular goitre was the most common lesion on thyroid FNA (62.50%) followed by follicular neoplasm and papillary carcinoma (18.75% each). In ascitic fluid samples, cytological diagnosis was benign in 12 (75%) cases and malignant in 4 (25%) cases. Cytological diagnosis in pleural fluid samples was benign in 12 (85.71%) cases and malignant in 2 (14.29%) cases. All this is evident from Table 2.

Table 2: Distribution of cases according to Cytological Diagnosis

Diagnosis	No. of cases N (%)
Breast (N=20)	
Fibroadenoma	11(55)
Fibrocystic disease	2(10)
Pyogenic abscess	2(10)
Ductal carcinoma	5(25)
Lymph Nodes (N=12)	
Reactive lymphadenitis	5(14.67)
Tubercular lymphadenitis	3(25)
Metastatic adenocarcinoma	4(33.33)
Salivary Gland (N=2)	
Pleomorphic adenoma	2 (100)
Thyroid (N=16)	
Nodular goitre	10(62.5)
Follicular neoplasm	3(18.75)
Papillary carcinoma	3(18.75)
Ascitic Fluid (N=16)	
Benign	12(75)
Malignant	4(25)
Pleural Fluid (N=14)	
Benign	12(85.71)
Malignant	2(14.29)

As summarised in Table 3, it was observed that maximum cumulative and mean score was obtained for MUF. The difference between the mean scores of H&E, Pap, MGG and MUF stains was statistically significant ($p < 0.05$) in all samples except salivary gland smears. MUF stain gives the best cytomorphological features with respect to the background, overall staining, cell morphology and nuclear characteristics in all the aspirates.

When the intergroup comparison was done between MUF and other stains in all FNA and body fluid samples, statistically significant difference was found between MUF and H&E, MGG and Pap stains as shown in Table 4 except in PAP vs MUF in lymph node, pleural and ascitic fluid.

Table 3: Relationship of mean scores of H&E, PAP, MGG & MUFP stains

Stain	Scores (Mean±SD)	p-value ANOVA test value)
Breast		
H&E stain	6.75 ±1.20	0.0001 (F=21.012)
Pap stain	8.35 ±0.87	
MGG stain	6.90 ±1.58	
MUFP stain	9.40 ±1.14	
Lymph Nodes		
H&E stain	7.58 ±0.99	0.0006 (F=6.882)
Pap stain	8.25 ±1.13	
MGG stain	7.33 ±1.07	
MUFP stain	9.16 ±1.11	
Salivary Gland		
H&E stain	6.50±0.70	-
Pap stain	8.00±0.00	
MGG stain	7.50±0.70	
MUFP stain	9.00±0.00	
Thyroid		
H&E stain	6.75±1.18	0.00001 (F=10.707)
Pap stain	8.06±1.28	
MGG stain	7.12±1.36	
MUFP stain	9.18±1.47	
Ascitic Fluid		
H&E stain	7.50±1.54	0.0144 (F=3.812)
Pap stain	8.56±1.15	
MGG stain	7.81±1.37	
MUFP stain	9.00±1.50	
Pleural Fluid		
H&E stain	7.28±1.48	0.0372 (F=5.6)
Pap stain	8.21±0.97	
MGG stain	7.64±1.54	
MUFP stain	8.57±1.55	

In our study, MUFP stain produced superior results with clear background, transparent cytoplasmic details, sharp nuclear features, and prominent nucleoli on both FNAC of various organs as well as on body fluids.

The maximum QI was obtained with MUFP stain, followed by Pap and MGG stains. QI with H&E stain was the least.

Discussion

Breast

In the present study, on 20 cases of breast aspirates, cytological diagnosis was comparable with that of other studies wherein the benign cases outnumbered the malignant cases. Similar results were also reported Shinde & Pandit [5] Choudhary et al. [6] also reported that MUFP stain is either equivalent to or better than the rapid Pap technique for cytological staining and study of various organs which is comparable to the present study.

Chan & Kung [7] in their study found that normal saline was the best rehydration fluid for air-dried smears providing blood-free background, which is comparable to the present study. Similar results were obtained by Choudhary et al and Arul et al [6,8] in their respective studies. It was seen that cells were larger in size because of air drying. Similar finding was observed by Chang & Kung [7] who reported that the cells were slightly larger in air-dried smears because of spreading of cells over larger areas. Yang et al., Yang & Hoda, Bandlish et al. and Shinde & Pandit [5,9-11] observed similar findings with MUFP.

In our study, it was observed that cell morphology was well seen. Nuclear details were excellent with open, vitreous chromatin in MUFP. There was no distortion of nuclear membrane. Yang and Alvarez [12] advised daily changing of alcoholic formalin. The solution is storage sensitive and a pH of 5 should be maintained. Prolonged immersion in this fixative affected cytomorphology and caused blurring.

Table 4: Comparison between MUFPP and H&E, PAP & MGG stains

Intergroup Comparison	Bonferroni 't' test	p-value
Breast		
H&E vs MUFPP	7.126	0.000
PAP vs MUFPP	3.263	0.002
MGG vs MUFPP	5.720	0.000
Lymph Nodes		
H&E vs MUFPP	3.669	0.001
PAP vs MUFPP	1.993	0.059
MGG vs MUFPP	4.105	0.000
Thyroid		
H&E vs MUFPP	5.166	0.000
PAP vs MUFPP	2.301	0.029
MGG vs MUFPP	4.119	0.000
Ascitic Fluid		
H&E vs MUFPP	2.777	0.009
PAP vs MUFPP	0.923	0.363
MGG vs MUFPP	2.328	0.027
Pleural Fluid		
H&E vs MUFPP	3.102	0.005
PAP vs MUFPP	1.391	0.176
MGG vs MUFPP	2.190	0.037

Table 5: Comparison of quality indices for H&E, PAP, MGG AND MUFPP stains in Fine Needle Aspirates of various sites in the study

Quality Indices for Stains	Values Obtained	
H&E	568/880	0.645
Pap	663/880	0.753
MGG	587/880	0.667
MUFPP	730/880	0.829

and wrinkling of nuclei. Similar observations were made by Kamal et al. and Sinkar & Arakeri [3,13]. In our study, we followed daily changing of alcoholic formalin and maintained it at a pH of 5.

In our study, nucleoli were distinctly seen and stained red in colour. Cytoplasm stained light green to blue and was transparent in MUFPP as compared to other stains. Kamal et al., Shinde & Pandit, Sinkar & Arakeri and Arul et al. [3,5,8,13] observed similar nuclear and cytoplasmic features with MUFPP stain.

Lymph Nodes

Shinde and Pandit [5] in their study found that benign lesions were more common than the malignant lesions, comparable to the present study. Khajuria et al [14] also had similar results in 80 lymph node aspirates. In lymph node aspirates maximum cumulative and mean score was obtained by MUFPP stain.

The cytomorphological features seen with MUFPP stain in the present study in lymph node aspirates were that the

background was clear, RBC-free even in blood mixed aspirates due to rehydration of air-dried smears by normal saline as shown in Figure 1. Chan & Kung and Shinde & Pandit [5,7] reported similar observations in their respective studies. Cell size was intermediate *i.e.*, smaller than those of MGG stained smears and larger than those of H&E and Pap stained smears. Chan & Kung, Shidham et al. and Shinde & Pandit [5,7,15] documented similar observations in their respective studies.

Cell morphology was well seen. Nuclear and nucleolar details were excellent, as seen in breast aspirates. Cytoplasm stained light green to blue and was transparent. These cytomorphological features were similar to those reported by Shinde & Pandit [5] in their study on lymph node aspirates using MUFPP stain. They observed that the correct diagnosis could not be made in three cases of metastatic squamous cell carcinoma. In these cases, malignant epithelial cells were easily identifiable, but keratinization of cytoplasm was not evident because of exclusion of Orange-G in the MUFPP stain.

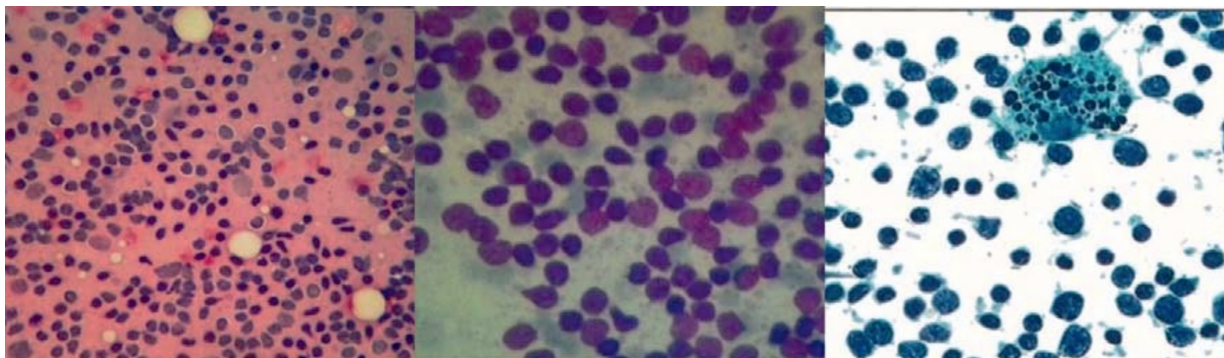


Figure 1: Reactive lymphadenitis - H&E*400 (left) & MGG*400 (center) smears show germinal center cells and lymphocytes with a hemorrhagic background in the former while MUF*400 (right) shows a mixed population of lymphoid cells and a tingible body macrophage with a nucleus showing open chromatin & prominent nucleoli in a clear background.

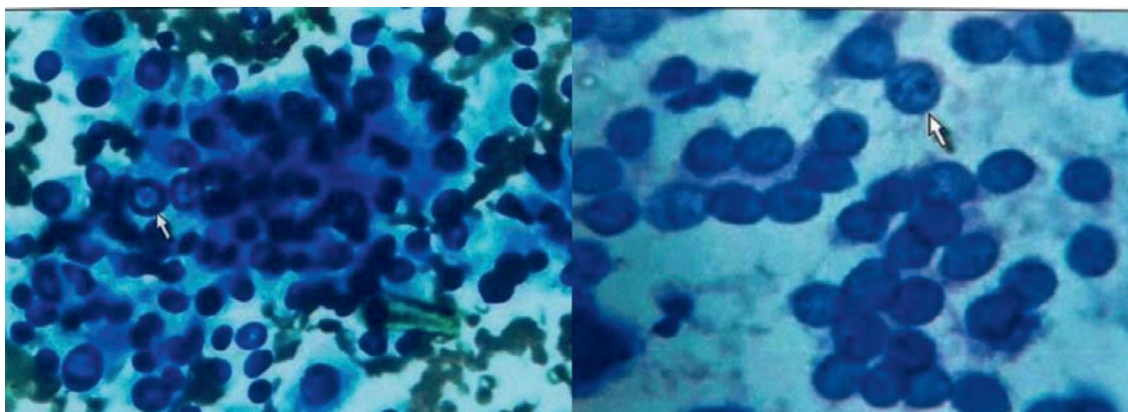


Figure 2: Papillary Carcinoma Thyroid - MGG*400 (left) shows increased cellularity & prominent intranuclear inclusions in a hemorrhagic background while MUF*1000 (right) in the same case shows nucleus with powdery chromatin & nuclear grooves in a clear background.

Arul et al. [8] in their study on comparative assessment of conventional Papanicolaou and modified ultrafast Papanicolaou stains in fine needle aspiration samples and body fluids similarly reported about quality of metastatic squamous cell carcinoma of lymph node as fair due to omission of Orange-G component, which led to difficulty in the identification of cytoplasmic keratinization.

Salivary Glands

The cytomorphological features seen after staining with MUF*P stain were similar as in breast and lymph node aspirates, with blood-free background and clear nuclear and cytoplasmic details. The results in the present study were comparable to those observed by Shinde & Pandit [5] who conducted their study on salivary gland aspirates using MUF*P stain.

Thyroid Gland

Thakur & Guttikonda [16] observed that MUF*P stain showed better staining pattern compared to other stains in thyroid swellings comparable to the present study. A clean blood-free background even in the blood mixed aspirates,

due to rehydration of smears with normal saline was seen in MUF*P staining in our study. Maruta et al. and Shinde & Pandit [5,17] in their respective studies on FNAC of thyroid nodule and various organs using MUF*P stain, observed that normal saline hemolyses RBCs and is therefore favourable to observe cell architecture and features, comparable to the present study. Sinkar & Arakeri [13] in their study observed that air-dried smears rehydrated within 30 minutes with normal saline for about 30 seconds provide cleaner and RBC free background compared to the wet fixed Pap smears.

Cell morphology was well seen. Nuclear chromatin was clear and well stained. In cases of papillary carcinoma of thyroid, powdery nuclear chromatin and intranuclear grooves were well appreciated in MUF*P as shown in Figure 2. Maruta et al. [17] in their study reported that addition of Gill-5 Haematoxylin into the Ultrafast procedure led to nuclear features being as clear as those of standard Pap stain, comparable with the present study. Shinde & Pandit [5] in their study observed that powdery nuclear chromatin and intranuclear grooves in cases of papillary carcinoma thyroid were well appreciated with MUF*P stain which correlates with our study as well. Cytoplasm stained light

green to blue and was transparent because of normal saline used in MUFP stain.

In the present study, colloid could not be seen in the background in cases of nodular goitre. Maruta et al. [17] observed that a minor disadvantage of MUFP stain as well as the original UFP stain, is the omission of colloid, which again is comparable to the present study.

Body Fluid

A clean blood-free background due to rehydration of air-dried smears by normal saline. Patrikar et al. [18] in their study on comparison of modified ultrafast Papanicolaou staining with standard Papanicolaou stain for fluids reported that the stain was useful in clearing the haemorrhagic background in malignant effusions.

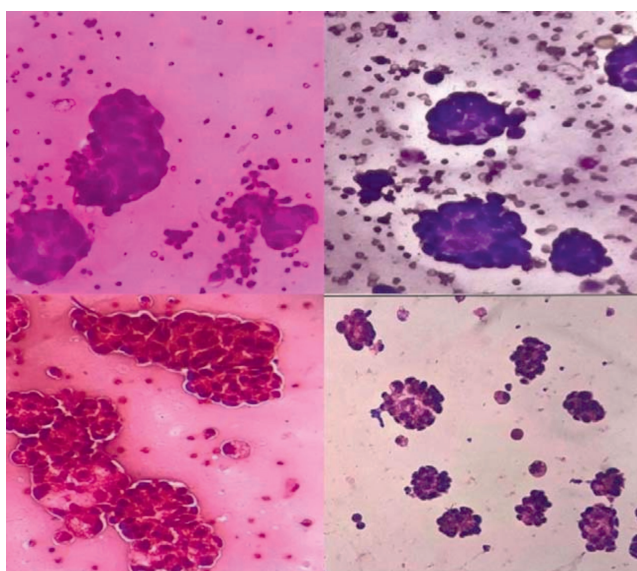


Figure 3: Ascitic fluid - H&E*400 smear (upper left) shows malignant cells with lymphocytes, MGG*400 smear (upper right) shows malignant cells with hyperchromatic nuclei & lymphocytes and PAP*400 smear (lower left) shows malignant cells in clusters in a hemorrhagic background while MUFP*400 smear (lower right) shows malignant cells arranged in clusters with crisp nuclear features in a clear background in the same case.

In MUFP, cell morphology was clear and nuclear details were well stained as shown in Figure 3. Yang et al. [19] in their study on application of Ultrafast Papanicolaou stain to body fluid cytology concluded that Ultrafast Papanicolaou stain improves the resolution of cytoplasmic and nuclear details of nonhematopoietic cells in body fluids. Patrikar et al. [18] in their study on comparison of Modified Ultrafast Papanicolaou staining reported that nuclear morphology was better highlighted in higher magnification. Arul *et al.* [8] in their study on comparative assessment of conventional Papanicolaou and Modified Ultrafast Papanicolaou stains in fine needle aspiration samples and

body fluids similarly reported that cell morphology and nuclear features of body fluids were comparable to conventional Pap stain. These observations were in agreement with the present study.

Limitations of the Study

- In this study only air-dried smears rehydrated with normal saline were used with MUFP as compared to alcohol fixed smears for staining.
- MUFP was not compared to other stains like toluidine blue etc which are used for on site evaluation.

Conclusions

Modified Ultrafast Pap (MUFP) stain with easy application in FNA and body fluid smears provides unequivocal cytomorphological details (clear nuclear and cytoplasmic features) that are superior to that provided with Pap, H&E and Giemsa stains. It can be used for checking the adequacy of material aspirated in FNAC and for rapid assessment and preliminary diagnosis of FNAC as well as body fluids due to short turnaround time though for latter, toluidine blue might be preferable. MUFP stain being rapid and reliable, is a very good alternative to Pap stain and other stains in benign and malignant lesions especially in developing countries like India.

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