COMPARATIVE STUDY OF MODIFIED ULTRAFAST PAPANICOLAOU STAIN AND RAPID ECONOMIC ACETIC ACID PAPANICOLAOU STAIN WITH THE ROUTINE STAINS USED IN CYTOLOGY

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ABSTRACT

BACKGROUND

Fine-Needle Aspiration Cytology (FNAC) has become a very useful technique in the diagnosis of mass lesions and it is the most preferred method as it provides early diagnosis, economical, less painful and is a procedure done in the outpatient department. The stains commonly used for FNAC smears are Papanicolaou stain (Pap), May-Grunwald Giemsa stain (MGG) and Haematoxylin and Eosin (H and E). The need to minimise time taken for staining has encouraged the development of newer techniques of staining. Ideally, these should be cost-effective without compromising on the quality of cell morphology.

MATERIALS AND METHODS

FNAC smears of 70 cases including 60 thyroid lesions and 10 salivary gland masses were analysed. A minimum of 5 smears were made in each case and stained with PAP, H and E, MGG, REAP and MUFP. Six parameters, namely the background, overall staining, cell morphology, nuclear characteristics, cytoplasmic details and air-drying artifacts were given scores to calculate quality index of each stain.

RESULTS

Age and sex distribution of all the cases. Thyroid lesions were more common in females (93.3%) and salivary gland lesions were common in males (60%).

CONCLUSION

In thyroid FNAC smears, PAP stain got the maximum quality index followed by H and E, REAP, MUFP and MGG. In salivary gland, Pap and H and E, both got the maximum quality index followed by REAP, MUFP and MGG. Therefore, REAP and MUFP can be included as routine stains in cytopathology when an earlier diagnosis is required.

KEYWORDS

Modified Ultrafast Papanicolaou Stain, Rapid Economic Acetic Acid Papanicolaou Stain.

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BACKGROUND

Cytopathology started as an innovative method for the early diagnosis by looking at imprints of cut surface of tumours and at post-mortem. It has evolved into a strong tool for faster and accurate diagnosis of mass lesions through new methods of procuring, fixing and staining cells. It has gained importance because of prompt assessment of cellular changes on material taken with minimally-invasive procedure and faster processing. The evolution of cytopathology happened through four overlapping eras. The early history (1860-1940); the development and expansion of exfoliative cytology (1940-1960); the consolidation of cytopathology as a discipline and the developments of population screening and FNA cytology (1955-1985); the maturation of cytopathology as a discipline and its integration with new technology (1985 to the present day).[1]

In the early historical era, microscopic features of normal and abnormal human cells in exfoliated or in imprints or scrapes were studied and recorded throughout the 19th century.[2,3] By first decade of 20th century, exfoliative cancer cells had been described in all types of specimens.[4] Dudgeon LS (1922) used cytology in at St. Thomas Hospital for the diagnosis of a wide variety of diseases from imprints of surgical specimens and he considered that the stained films were better to examine than paraffin sections.[5,6] At the same time in USA, FNA cytology was being developed and the first series on aspiration of neoplasms was published from Memorial Hospital for Cancer and Allied Diseases in New York City.[7]

A second era of cytopathology began in 1941 with the publication of the diagnostic value of vaginal smears in carcinoma of uterus by George N. Papanicolaou, an anatomist and Herbert F. Traut, a gynaecologist.[8]

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Papanicolaou is known as "the father of cytopathology" and his contributions are most important regarding wet fixation in cytology and systematic observation of cancer cells, the details of which were presented in his paper on New Cancer Diagnosis.[8,9] The Papanicolaou stain (Pap) is a multichromatic staining technique named after Papanicolaou who developed it in 1942 and subsequently modified in 1954 and in 1960 and is used extensively in both exfoliative and FNA cytology.[10,11] As quick diagnosis is essential in cytology, there are a few rapid stains available, which include MGG stain, Diff-Quik stain and toluidine blue stain. However, cytopathologists prefer the traditional, transparent, crisp nuclear features offered Pap stain rather than air-dried smears.[12]

Ultrafast Papanicolaou Stain (UFP) was introduced by Yang and Alvarez in 1994, which is a hybrid of Romanowsky stain and Pap stain. It not only reduces the time for Pap stain to 90 seconds, but also enhances the quality.[13] UFP stain has its limitation like the reagents required for the procedure are not universally available and the advantages include cells appear larger with clear background and bring out vibrant colours in the cells.

Pap stain requires 95% ethanol for fixation, which is expensive and laboratory needs a license for acquiring it in bulk quantity. To overcome this, REAP staining method, which is rapid and less expensive was developed by the Department of Pathology of Tata Memorial Hospital. In this method, smears are prefixed in methanol and 95% ethanol is replaced by 1% acetic acid.[14]

Aims and Objectives- The objectives of the present study was to compare the results of two faster methods of staining namely, Modified Ultrafast Papanicolaou stain (MUFP) and Rapid Economic Acetic Acid Papanicolaou stain (REAP) with routine Pap, H and E and MGG stains.

MATERIALS AND METHODS

This was a prospective study done during the period of August 2012 to July 2013. Study included fine-needle aspiration material from mass lesions of thyroid and salivary gland. Sample size was determined based on Shinde et al[15] and Chaudhury et al.[16] Total number of cases studied were 70, which included 60 cases of thyroid lesions and 10 cases of salivary gland. The FNA procedure was performed after informed consent was taken from the patient along with clinical history and examination. In case of thyroid, mainly fine-needle non-aspiration method was used and for all the salivary lesions aspiration was done by standard method. For each case, minimum five smears were made on clean glass slides of which 2 smears were fixed in 95% ethanol for at least 15 minutes. These smears were submitted for Pap and H and E stain. For REAP stain, smears was fixed in methanol and at least two smears were air dried, out of which, one was stained by MGG stain and other smear was rehydrated with normal saline and subsequently fixed in alcoholic formalin and stained by MUFP stain. The procedures followed for MUFP and REAP are given below.

Modified Ultrafast Papanicolaou Stain-

1.	Normal saline	30 seconds
2.	Alcoholic formalin	10 seconds
3.	Water	6 slow dips
4.	Gill's Haematoxylin	2 slow dips
5.	Water	6 slow dips
6.	95% ethanol	6 slow dips
7.	EA-50	4 slow dips
8.	95% Ethanol	6 slow dips
9.	100% Ethanol	6 slow dips
10.	Xylene	10 slow dips

Rapid Economic Acetic Acid Pap Stain-

Smears are fixed in methanol for 30 minutes.

1.	1% acetic acid	10 dips.
2.	Harris Haematoxylin, preheated 60°C	10 dips.
3.	Tap water	10 dips.
4.	1% acetic acid	10 dips.
5.	OG-6	10 dips.
6.	1% acetic acid	10 dips.
7.	EA-50	10 dips.
8.	1% acetic acid	10 dips.
9.	Methanol	10 dips.
10.	Xylene	10 dips.

Blotting was done after each step and all the smears stained were coverslipped. The stained smears were analysed and a scoring method was adopted pertaining to six parameters and addition of these scores for each type of stain was done. The maximum score for a single case taking into account all the six parameters was 17. The "Quality Index" was obtained by calculating the ratio of actual score obtained to the maximum score possible.

Quality index = Actual score obtained/maximum score possible.

Quality index for each of the five stains of the two organs was compared and analysed.

Parameters	Score=1	Score=2	Score=3
Background	Haemorrhage	Clean	
Overall staining	Poor	Average	Good
Cell morphology	Poorly preserved	Moderately preserved	Well preserved
Nuclear characteristics	Smudgy chromatin	Moderately crisp chromatin	Crisp chromatin
Cytoplasmic details	Unsatisfactory	Suboptimal	Optimal
Air-drying artifacts	>50%	<50%	0%

Table 1. Scoring System Used in the Assessment of Staining

Statistical Analysis

Descriptive and inferential statistical analysis has been carried out in the present study. Fisher exact test has been used to find the significance of study parameters on categorical scale between two or more groups. The Statistical software namely SAS 9.2, SPSS 15.0, Stata 10.1, MedCalc 9.0.1, Systat 12.0 and R environment version 2.11.1 were used for the analysis of the data.

RESULTS

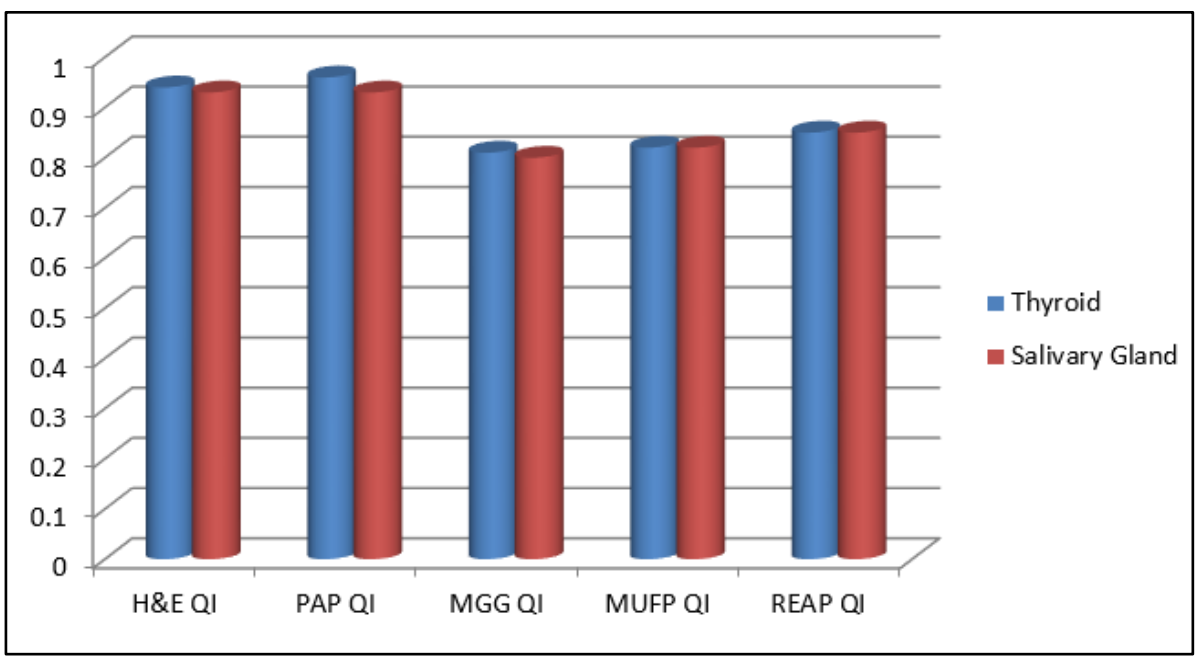
Age and sex distribution of all the cases were as follows. Thyroid lesions were maximum in the age group of 31-40 (40%) followed by 21-30 years (23.3%). Distribution of cases in the salivary gland is maximum in age group of 31-40 (30%) and 51-60 (30%) years. Thyroid lesions were more common in females (93.3%) and salivary gland lesions were common in males (60%).

Mean Quality Index of Two Organs

Quality index were calculated for all the smears stained with five stains and mean was calculated.

Organ	Cases	H and E	PAP	MGG	MUFP	Reap
Thyroid	60	0.94	0.96	0.81	0.82	0.85
Salivary gland	10	0.93	0.93	0.80	0.82	0.85

Table 2. Mean Quality Index of Five Stains in all Cases of Thyroid and Salivary Gland Lesions



Graph 1. Quality Index score obtained for thyroid and salivary gland FNAC smears with PAP, H&E, REAP, MUFP and MGG.

PAP- Papanicolaou stain,
H and E- Haematoxylin and Eosin stain,
MGG- May-Grunwald Giemsa stain,
MUFP- Modified Ultrafast Papanicolaou stain,
REAP- Rapid Economic Acetic Acid Papanicolaou stain and
QI - Quality Index.

In thyroid, PAP stain got the maximum quality index score followed by H and E, REAP, MUFP and MGG.

In salivary gland, PAP and H and E stain got the maximum quality index score followed by REAP, MUFP and MGG.

Mean quality index of the five stains with all cases of thyroid is given in Table 3.

Thyroid Lesions

	H and E	PAP	MGG	MUFP	REAP
Background					
• Haemorrhagic	23 (38.3%)	18 (30%)	49 (81.7%)	14 (23.3%)	44 (73.3%)
• Clean	37 (61.7%)	42 (70%)	11 (18.3%)	46 (76.7%)	16 (26.7%)
Overall Staining					
• Poor	0 (0%)	0 (0%)	2 (3.3%)	0 (0%)	0 (0%)
• Average	2 (3.3%)	1 (1.7%)	39 (65%)	36 (60%)	24 (40%)
• Good	58 (96.7%)	59 (98.3%)	19 (31.7%)	24 (40%)	36 (60%)
Cell Morphology					
• Poorly preserved	0 (0%)	0 (0%)	1 (1.7%)	1 (1.7%)	0 (0%)
• Moderately preserved	4 (6.7%)	1 (1.7%)	19 (31.7%)	31 (51.7%)	16 (26.7%)
• Well preserved	56 (93.3%)	59 (98.3%)	40 (66.7%)	28 (46.7%)	44 (73.3%)
Nuclear Characteristics					
• Smudgy chromatin	0 (0%)	0 (0%)	1 (1.7%)	0 (0%)	0 (0%)
• Mod crisp chromatin	5 (8.3%)	1 (1.7%)	35 (58.3%)	42 (70%)	14 (23.3%)
• Crisp chromatin	55 (91.7%)	59 (98.3%)	24 (40%)	18 (30%)	46 (76.7%)
Cytoplasmic Details					
• Unsatisfactory	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
• Suboptimal	3 (5%)	1 (1.7%)	20 (33.3%)	35 (58.3%)	20 (33.3%)
• Optimal	57 (95%)	59 (98.3%)	40 (66.7%)	25 (41.7%)	40 (66.7%)
Air-Drying Artifacts					
• >50%	0 (0%)	0 (0%)	3 (5%)	1 (1.7%)	0 (0%)
• <50%	21 (35%)	21 (35%)	13 (21.7%)	19 (31.7%)	30 (50%)
• 0%	39 (65%)	39 (65%)	44 (73.3%)	40 (66.7%)	30 (50%)

Table 3. Results of 60 Thyroid Lesions According to Scores given in Six Parameters to find Quality Index for each of the Five Stains

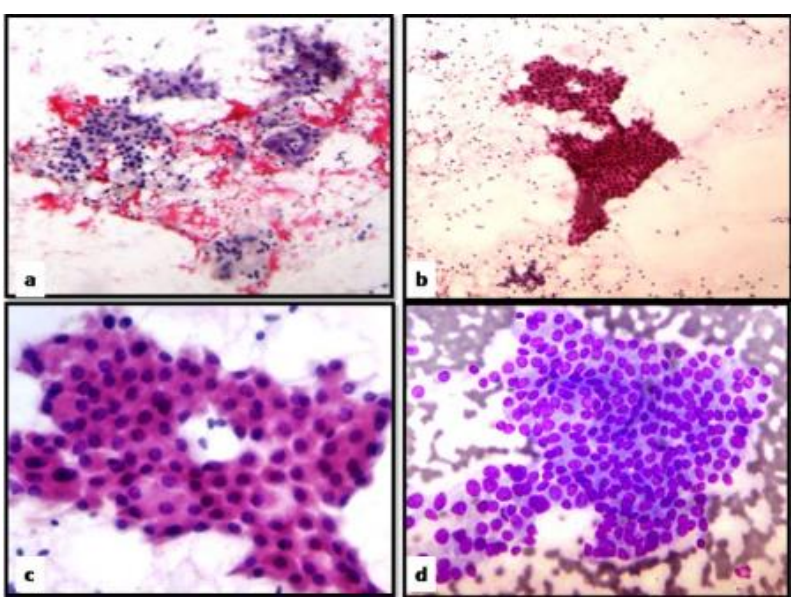


Figure 1. Hashimoto's Thyroiditis, a) Pap Stain, 100x, b) H and E Stain, 100x, c) H and E Stain, 400x, d) MGG Stain, 400x

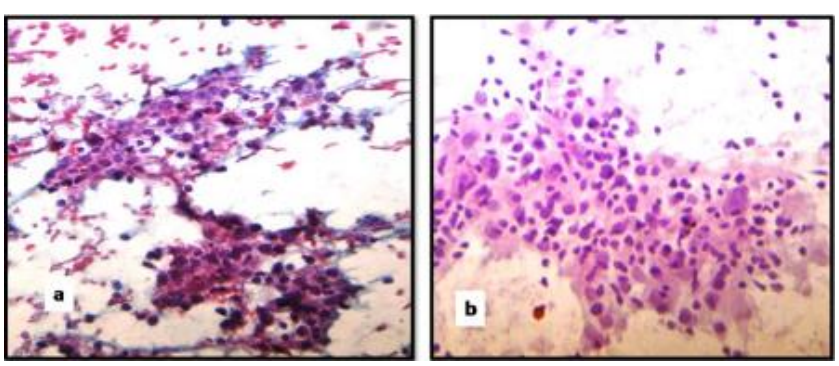


Figure 2. Hashimoto's Thyroiditis, REAP Stain, 400x and b) MUFP Stain, 400x

Salivary Gland (10 Cases)

	H and E	PAP	MGG	MUFP	REAP
Background					
• Haemorrhagic	7 (70%)	7 (70%)	9 (90%)	2 (20%)	6 (60%)
• Clean	3 (30%)	3 (30%)	1 (10%)	8 (80%)	4 (40%)
Overall Staining					
• Poor	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
• Average	1 (10%)	1 (10%)	8 (80%)	5 (50%)	5 (50%)
• Good	9 (90%)	9 (90%)	2 (20%)	5 (50%)	5 (50%)
Cell Morphology					
• Poorly preserved	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
• Moderately preserved	0 (0%)	0 (0%)	2 (20%)	5 (50%)	3 (30%)
• Well preserved	10 (100%)	10 (100%)	8 (80%)	5 (50%)	7 (70%)
Nuclear Characteristics					
• Smudgy chromatin	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
• Mod crisp chromatin	0 (0%)	0 (0%)	9 (90%)	9 (90%)	2 (20%)
• Crisp chromatin	10 (100%)	10 (100%)	1 (10%)	1 (10%)	8 (80%)
Cytoplasmic Details					
• Unsatisfactory	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
• Suboptimal	0 (0%)	0 (0%)	1 (10%)	5 (50%)	2 (20%)
• Optimal	10 (100%)	10 (100%)	9 (90%)	5 (50%)	8 (80%)
Air-Drying Artifacts					
• >50%	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
• <50%	4 (40%)	4 (40%)	4 (40%)	4 (40%)	7 (70%)
• 0%	6 (60%)	6 (60%)	6 (60%)	6 (60%)	3 (30%)

Table 4. Results of 10 Salivary Gland Lesions According to Scores given in Six Parameters to Find Quality Index for each of the Five Stains

PAP- Papanicolaou stain.
H and E- Haematoxylin and Eosin stain.
MGG- May-Grunwald Giemsa stain.
MUFP- Modified Ultrafast Papanicolaou stain.
REAP- Rapid Economic Acetic Acid Papanicolaou stain.

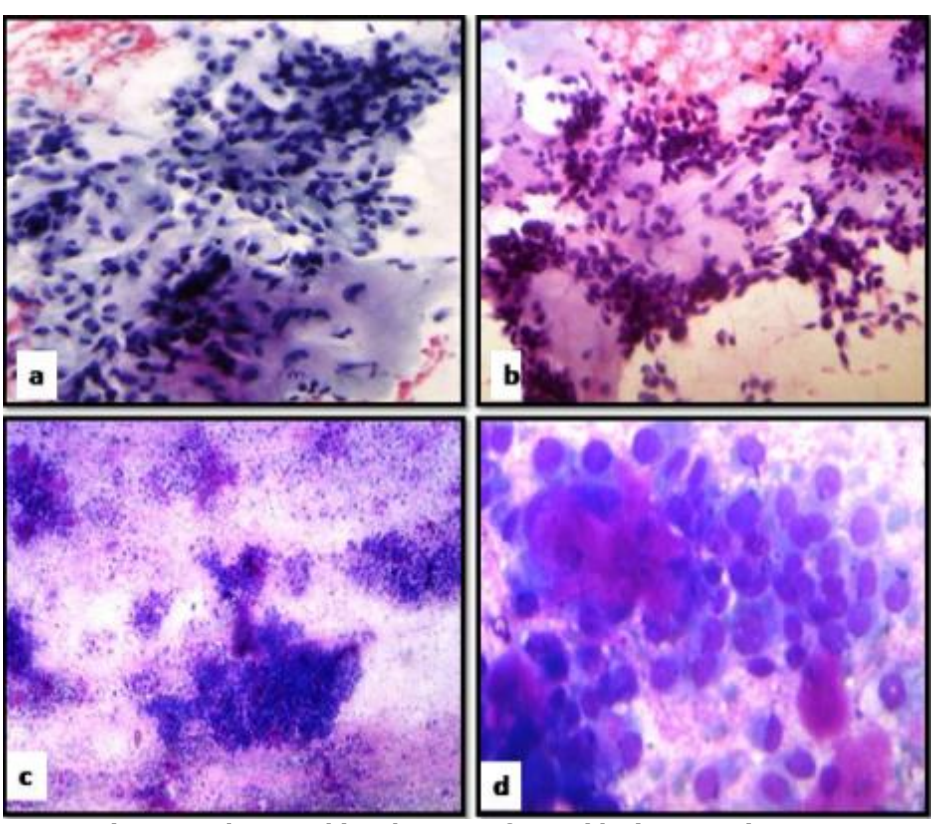


Figure 3. Pleomorphic Adenoma of Parotid, a) Pap Stain, 400x, b) H and E Stain, 400x, c) MGG Stain, 100x and d) MGG Stain, 400x

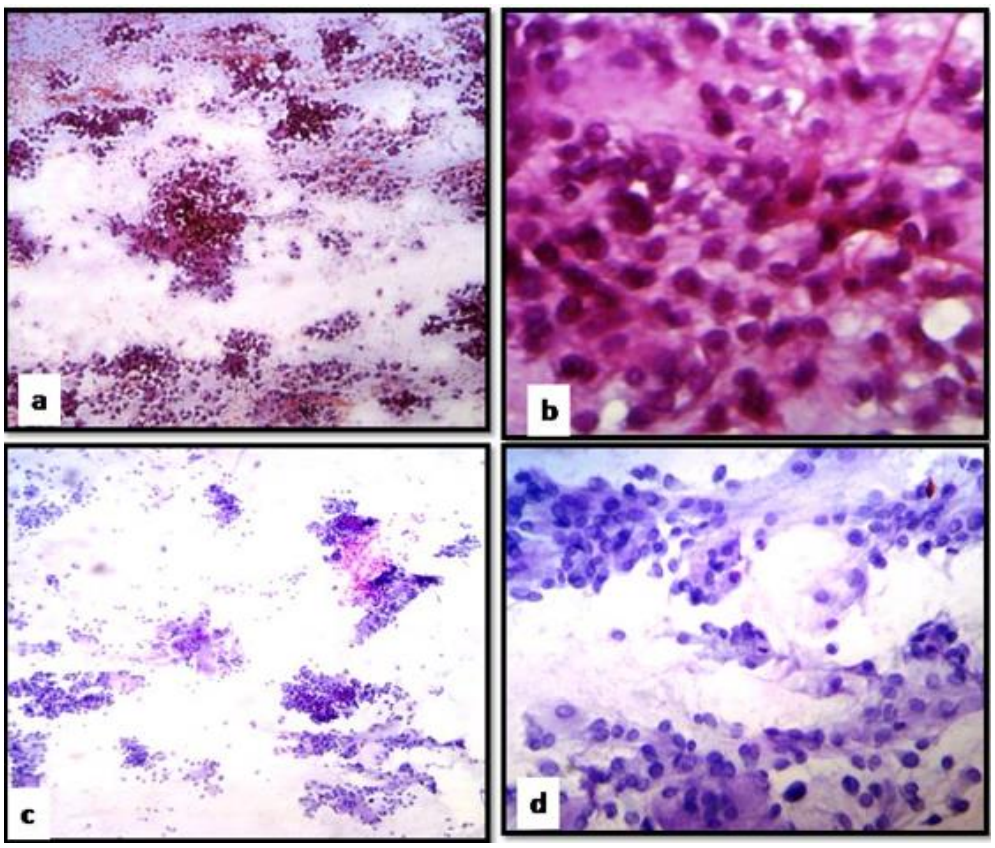


Figure 4. Pleomorphic Adenoma of Parotid, a) REAP Stain, 100x and b) 400x, c) MUFP Stain, 100x and d) MUFP Stain, 400x

Figure.1 and figure.2 show the cytological features of all the five stains used with their characteristic parameters used to calculate quality index. Similarly figure.3 and 4 show the cytological features of the same stains used for salivary gland lesions.

Results of Specific Stain in Two Organs-

Mean quality index score for H and E stain is maximum for thyroid followed by salivary gland.

Mean quality index score for Pap stain is maximum for thyroid followed by salivary gland.

Mean quality index score for MGG stain is maximum for thyroid followed by salivary gland.

Mean quality index score for MUFP stain is maximum for thyroid followed by salivary gland.

Mean quality index score for REAP stain is maximum for thyroid followed by salivary gland.

DISCUSSION

FNAC was first introduced in Sweden by Franzen, a haematologist and oncologist by training who used the same Romanowsky staining method as for bone marrow aspirates. Presently, FNAC relies on obtaining a satisfactory specimen on which a reliable diagnosis can be made and is an established method as the first line investigation of mass lesions. FNAC is one of the less expensive (economical), fastest and easiest tools available for early detection and diagnosis of various lesions. Since its inception, PAP stain remains the traditional and preferred stain for the gynaecological cytology and also for the lesions of other organs. The different stains used for air-dried smears, such as May-Grunwald-Giemsa, Jenner Giemsa and Diff-Quick fail to offer the transparency for the study of subtle nuclear features as seen by the PAP stain.

The traditional pap stain involves wet fixation with expensive ethanol and subsequent staining together requiring at least 30 minutes. To cut down the time, the rapid pap stains were developed by Kline, Tao and Sato with respective staining time of 4 minutes, 5 minutes and 90 seconds. However, the quality of rapid Papanicolaou staining is usually not satisfactory as the cell morphology is not well seen. To overcome these problems, Yang and Alvarez developed Ultrafast Pap (UFP) stain, which is a hybrid of Papanicolaou and Romanowsky stains. The staining time of UFP is 90 seconds.[14] Kamal et al from India further modified the UFP stain (modified ultrafast pap stain) to overcome the problem of shortage of Richard-Allan haematoxylin and Richard-Allan Cyto-Stain in Indian setup. This method has a short staining time of 130 seconds and the cytomorphology can be well appreciated.[17] Air-dried smears can be easily transported also to a distant laboratory.

In the present study, cytomorphology of rehydrated air-dried smears followed by modified ultrafast Papanicolaou staining was compared with methanol fixed smear stained by REAP stain and ethyl alcohol fixed smears stained by conventional pap stain, H and E stain and air-dried smears stained by MGG.

The quality of all 5 stains was evaluated on 6 parameters such as background, overall staining, cell morphology, nuclear characteristics, cytoplasmic features and air-drying artifacts.

Of all the cytological stains we did for thyroid and salivary gland lesions, highest quality index score was seen in Pap stain followed by H and E, REAP, MUFP and MGG, which was comparable with other studies.[18,19]

Shinde et al calculated quality index of four sites, lymph node, breast, thyroid and salivary gland for ultrafast pap stain as follows. The quality indices in this study are comparable to our study.

Organ	Shinde's Study		Present Study	
	No. of Cases	Quality Index	No. of Cases	Quality Index
Thyroid	8	0.98	60	0.82
Salivary gland	2	0.95	10	0.82

Table 5. Quality Index in Different Organs in Shinde's Study and Present Study for MUFP

CONCLUSION

Papanicolaou stain is excellent in studying FNAC material of two organs, namely thyroid and salivary gland lesions. It has the maximum score in all five parameters except for air-drying artifacts. H and E has good score, but still is not as good as conventional Pap stain.

MGG showed less air-drying artifacts when compared to wet-fixed smears. REAP stain is as good as Papanicolaou stain. It is economical and staining time is also lesser. The nuclear characteristics are crisp in REAP, which is comparable with that of Pap stain. MUFP-stained smears showed clean background and less air-drying artifacts, takes lesser time for fixation and makes nucleoli red and prominent. Air-drying artifacts are less in air-dried smears like MGG and air-dried, rehydrated smears as in MUFP compared to wet-fixed smears. MUFP is a very good stain for inflammatory lesions.

This study emphasises that REAP and MUFP stains can be used as routine cytological stains.

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