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TO COMPARE THE EFFICACY OF RAPID ECONOMIC ACETIC ACID PAPANICOLAOU STAIN (REAP) WITH CONVENTIONAL PAPANICOLAOU STAIN CERVICAL CYTOLOGY

Pathology

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ABSTRACT

Introduction- The Papanicolaou (pap) stain is a technique of cytological staining. . PAP stain is being used in screening programme. The aim of Pap Stain is to decrease the mortality by detecting cervical pre-cancerous condition. HPV infection is the main cause of cervical cancer. Cervical screening is being performed for more than 50 years by conventional method of Pap staining. Dighe et al replaced alcohol with 1% acetic acid, as acetic acid was easily available and cheaper. This rapid method was named as rapid, economic, acetic acid Papanicolaou (REAP) stain. **Aim-** To study cytomorphological parameters, smear preservation and turnaround time of conventional Pap and REAP. To compare the efficacy of REAP preparations with conventional Pap and classify as per Bethesda System of Cervical Cytology (2014) **Material and Methods-** A prospective study of cervical smears from 500 patients conducted in Department of Pathology referred from the Department of Obstetrics & Gynaecology, SGT University during period December 2018 to August 2020. **Results-** Cytomorphological parameters, smear preservation and turnaround time of conventional Pap and REAP were compared by calculating the p-Value. **Conclusion-** REAP is a simple, easy and cheaper staining method with good cytomorphological features over conventional staining. REAP has a very simple and rapid technique with good smear preservation, can easily be followed in source limited areas. Hence in view of all the advantages, it would be worthwhile to use this technique for rapid staining.

KEYWORDS

Cervical, Conventional, Cytomorphological, Papanicolaou, REAP.

INTRODUCTION

The Papanicolaou (pap) stain is a technique of cytological staining. George Papanicolaou introduced this technique in 1942 and again modified in 1954 and 1960. It decreases the mortality and morbidity of cervical cancer due to early diagnosis and treatment of various precancerous condition of cervix.[1] Human Papilloma Virus (HPV) is associated with nearly 99% cases of cervical carcinoma. High risk HPV are 16,18,31,33,35,39,45, 51,52,56,58,59,68,73 and 82. Out of these 16 and 18 cause 70% of worldwide cervical cancer.[2] Chlamydia also causes mucosal injury which increases chances of HPV infection.[3] In developing countries like India, cervical cancer is the leading cause of death in women as compared to developed countries. So screening should be done by Pap smear to reduce the death rate[4]. Pap smear is used for early detection of cervical cancer as this test is cheap, easy and widely accepted.[5]

Cervical cancer cell was first noticed by Papanicolaou in 1925. In 1942, he described 5-dye protocol and finally presented in 1960 with better visualization of both nuclear and cytoplasmic details in squamous cell maturation.[6,7] As per American Cancer Society (ACS), Pap smear should be started at the age of 21 years for every three years. At 30 and older it should be done after every three years if Pap smear alone is normal and every 5 years if Pap smear along with HPV co-testing. Screening should be stopped at the age of 65 years if there is adequate screening and no history of CIN-2, CIN-3 or adenocarcinoma-in-situ or cervical cancer during last 10 years. If a woman had total hysterectomy stop cervical cancer screening.[8]

From many years, the conventional Pap smear is being used for early diagnosis and treatment of cervical carcinoma. In Conventional Pap staining, ethanol is used as dehydrating agent in large amount which is costlier and which requires license for the purchase of large amount of alcohol. This is a longer procedure and takes about 20 minutes.[9]

Dighe et al replaced alcohol with 1% acetic acid, as acetic acid was easily available and cheaper. This rapid method was named as rapid, economic, acetic acid Papanicolaou (REAP) stain. On comparing, the cytoplasmic and nuclear morphological features of REAP and conventional Pap smear, there was difference in transparency of cytoplasmic and nuclear morphology of REAP Stain and conventional stain.[10,11,12]

Various advantages of REAP over conventional Pap stain:[13]

- Cytoplasmic and nuclear staining intensity is better.
- It is rapid, takes only 7 minutes.
- It is cost effective.
- The stained smear with REAP shows long term preservation of colour.

MATERIAL AND METHODS

Source of Data: A prospective study of cervical smears from 500 patients was conducted in the Department of Pathology referred from the Department of Obstetrics & Gynaecology, SGT Medical College.

2 smears were taken from each patient sent in a container containing ethanol, one stained by conventional and other by rapid economic acetic acid Pap (REAP) for the reporting of Cervical smear according to Bethesda System of Classification.

Study period: From December 2018 to August 2020 after clearance from ethical committee.

Inclusion criteria: Cervical cytology samples from women of age group 21-65 years attending Obstetrics & Gynaecology Out Patient Department with complaints of:-

1) White discharge per vagina
2) Post-coital bleeding
3) For routine cervical cancer screening.

Exclusion criteria:

1. Pregnant women.
2. Patient with massive bleeding per vagina.
3. Cervical carcinoma cases on radiation therapy.

Conventional PAP technique[1]

- Transfer slides from alcohol-ether fixative without drying to 80% alcohol to 70% and 50% alcohol to distilled water.
- Dip in Harris hematoxylin for 4 minutes.
- Rinse in distilled water.
- Dip in 0.25% HCl in 50% ethanol about six times (20-60 sec).
- Place in scotts tap water/Bluing (magnesium sulphate 30.0 gm, sodium bicarbonate 2.0 gm and tap water 3000.0 ml) for 6 minutes.
- Rinse in distilled water and run through 50%, 70%, 80% and 95% alcohol.
- Dip in OG-6 for 2 minutes.
- Rinse in two changes of 95 % alcohol.

- Dip in EA36/EA 50 for 2 minutes.
- Rinse in three changes of 95% alcohol. Dehydrate in absolute alcohol, followed by equal parts absolute alcohol and xylol and mount.

REAP staining technique[1]
- 1% acetic acid 10 dips.
- Harris Haematoxylin, preheated 60°C 10 dips.
- Tap water 10 dips.
- 1% acetic acid 10 dips.
- OG-6 10 dips.
- 1% acetic acid 10 dips.
- EA-50 10 dips.
- 1% acetic acid 10 dips.
- Methanol 10 dips.
- Xylene 10 dips.

Bethesda system of classification of cervical cytology 2014.[14]
Squamous cells as
1) Atypical Squamous Cells
- Of Undetermined Significance (ASC-US)
- Cannot exclude HSIL (ASC-H)

2) Squamous Intraepithelial Lesion(SIL)
- Low grade Squamous Intraepithelial Lesions (LSIL)/ CIN1
- High grade Squamous Intraepithelial Lesions (HSIL)
- Moderate Dysplasia/ CIN2
- Severe Dysplasia/ CIN3
- Carcinoma in situ/ CIN3

3) Squamous cell carcinoma
Glandular cells as
1) Presence of endometrial cells in
- Out-of-phase in menstruating woman
- In postmenopausal woman
- No menstrual history available

2) Atypical
- Endocervical cells
- Endometrial cells
- Glandular cells

3) Adenocarcinoma

RESULTS
The maximum number of women presented were in age group of 31-40 years that is 197 (39.4%) and least that is 18 (3.6) of >60 years of age group. Mean age of women are 38.34 ± 10, mean age with standard deviation (21-65yrs).

The cases are divided into normal and abnormal categories. The normal cases includes screening of cervical lesions, inflammatory smears, infectious smears like bacterial vaginosis, candidiasis, trichomonal vaginitis and atrophic smears.

Abnormal smears included ASCUS, ASC-H, LSIL, HSIL, SCC, AGC-NOS, AEC and adenocarcinoma.

56 (11.2%) cases were asymptomatic and maximum number of patients with white discharge per vaginum i.e, 171 (34.2%) out of which 6 cases were diagnosed with epithelial cell abnormalities and 165 cases were within normal limits.95 cases presented with blood stained discharge out of which 4 cases were found to be abnormal and 91 cases were normal.

82 cases presented with itching and pain per vaginum, 6 were found abnormal and 76 were normal.4 cases (0.8%) presented with postcoital bleeding, out of these 3 were epithelial abnormal and 1 normal smear.(Graph 1)

Out of 500 cases, 383 (76.6%) smears were satisfactory for evaluation with adequate cellularity. 96 (19.2%) smears were satisfactory for evaluation with fair cellularity but limited vision due to partial obscuring by blood and inflammation whereas 21 smears (4.2%) were unsatisfactory for evaluation.(Table 1, Graph 2).

With conventional stain, 464 (92.8%) smears showed distinct and 36 (7.2%) smears showed indistinct cytoplasmic border. 428 (85.6%) smears were satisfactory and 72 (14.4%) were unsatisfactory for cytoplasmic staining. The nuclear borders were distinct in 464 (92.8%) cases and indistinct in 36 (7.2%) cases. The chromatin staining was distinct in 465 (93%) cases and hazy in 35 (7%) cases. With REAP stain, 472 (94.4%) smears showed distinct and 28 (5.6%) smears showed indistinct cytoplasmic border. 448 (89.6%) smears were satisfactory and 52 (10.4%) were unsatisfactory for cytoplasmic staining. The nuclear border was distinct in 471 (94.2%) cases and indistinct in 29 (5.8%) cases. The chromatin staining was distinct in 473 (94.6%) cases and hazy in 27 (5.4%) cases. The p-value for cytoplasmic border, cytoplasmic staining, nuclear border and chromatin staining are 0.01259, 0.00569, 0.03720 and 0.02765 respectively. All are statistically significant (<0.05) (Table 2, Graph 3). The optimal cytoplasmic and nuclear staining with REAP stain were 448 (89.6%) and 466 (93.2%) respectively. With conventional stain, optimal cytoplasmic staining was in 428 (85.6%) cases and optimal nuclear staining was in 441 (88.2%) of cases. The smear preservation is good in both techniques even after 1 year. The p-value for the cytoplasmic and nuclear staining is statistically significant (<0.05). The staining time was 20 minutes with conventional method and 7 minutes with REAP (Table 3, Figure 4,5).

Out of total 500 cases, 452 cases were diagnosed as NILM, 21 cases with epithelial abnormality, 6 cases with glandular cell abnormality and 21 cases were unsatisfactory for evaluation with conventional stain. With REAP stain, 452 cases were diagnosed as NILM, 22 cases with epithelial cell abnormality, 6 cases with glandular cell abnormality and 21 cases were unsatisfactory for evaluation.(Table 4, Graph 6)

Out of 451 (90.2%) reported as NILM on conventional stain, 305 (61%) cases were inflammatory or reactive, 54 (10.8%) were without any inflammation, 40 (8%) were atrophic, 41 (8.2%) were reported as bacterial vaginosis, 9 (1.8%) were with candidial infection and 3 (0.6%) were of trichomonal vaginitis whereas on REAP stain, among 452 NILM cases, 306 (61.2%) cases were inflammatory or reactive, 54 (10.8%) were without any inflammation, 40 (8%) were atrophic, 41 (8.2%) were reported as bacterial vaginosis, 8 (1.6%) were with candidial infection and 2 (0.4%) were of trichomonal vaginitis.(Table 6, Graph 7)

Out of 500 cases, 21 (4.2%) cases were diagnosed for epithelial cell abnormality, 6 (1.2%) cases were of ASCUS on conventional. Out of 22 (4.4%) cases diagnosed as epithelial cell abnormality on REAP, whereas ASCUS 7 (1.4%), ASC-H 4 (0.8%), LSIL 4 (0.8%), 5 (1%) HSIL and 2 were SCC on both conventional and REAP stain (Table7, Figure 8).

6 (1.2%) caseswere found with glandular cell abnormality out of which 4 (0.8%) cases were of atypical glandular cell, NOS and 2 (0.4%) were of atypical endocervical cell on both conventional and REAP stain (Table 8, Figure 9).

Results with conventional stain and REAP were compared as per Bethesda System of Cervical Cytology 2014 (Table 9, Figure 10).

DISCUSSION
In present study out of total 500 cases majority i.e. 197 (39.4%) were in 31-40 years of age group which is comparable to study done by Deshpande et al[15] and Vani et al[16]. The most common presenting complaint in present study was white discharge per vaginum in 171 cases (34.2%) which is comparable to study by Sachan et al[17] which had vaginal discharge in 36.96% cases followed by blood stained discharge in 95 cases (19%). Maximum number of cases i.e. 6 cases each were associated with epithelial cell abnormality were associated with chief complaints of white discharge per vaginum and itching, pain per vaginum. 4 cases presented with post coital bleeding out of which 3 had epithelial cell abnormality.

In this study, the main aim was to compare the cytoplasmic and nuclear staining of REAP with conventional stain and then categorize as per "The Bethesda System of Cervical Cytology." It is observed that out of 500 cases, 448 cases showed optimal cytoplasmic staining on REAP, which were 428 on conventional Pap stain. The remaining 52 cases showed the suboptimal staining may be due to due to thick smear or poor preservation. The nuclear features were crisp and optimal in 466 cases on REAP stain and 441 on conventional stain. The rest of 34 cases were suboptimal on REAP and 59 cases on conventional stain.

This is comparable to a study done by Hussein et al[17] to compare modified Rapid Papanicolaou stain i.e. REAP as an alternative to standard Pap stain .76 smears were compared out of which 62 cases (82%) were optimal for cytoplasmic stain and 69 cases (90%) for nuclear stain on REAP. Biswas et al[18] performed similar study on 110 patients out of which 100 and 105 cases were optimal for cytoplasmic and nuclear staining respectively.

Dighe et al[15] conducted a study to compare this rapid and economical staining method with conventional Pap stain in which total 200 patients were taken. From each patient 2 smears were stained with these techniques and results were compared .Out of 200 cases, 181(92%) cases showed optimal cytoplasmic staining and 192(96%) showed optimal nuclear staining. Suboptimal staining is probably due to poor penetration of stain in large cell clusters.

Deshpande et al[16] studied 200 cases to compare the cytomorphological features on REAP with conventional Pap stain .Out of 200 cases, 99% and 98% of cases have distinct cytoplasmic border on conventional and REAP stain respectively. Cytoplasmic stain was satisfactory in 99% and 98% cases on conventional and REAP stain respectively. Distinct nuclear border and distinct chromatin staining was in 99% and 98% cases respectively. All the features were comparable on both staining techniques.

Vani et al[20] studied 100 cases, 98% cases had distinct cytoplasmic borders, 97% cases had satisfactory cytoplasmic staining, 96% cases with distinct nuclear border and 97% with distinct chromatin staining on REAP stain whereas on conventional stain 95% had distinct cytoplasmic borders, 96% cases had satisfactory cytoplasmic staining, 97% cases with distinct nuclear border and 95% with distinct chromatin staining. Results were comparable with our study.

Gupta et al[26] studied 480 cases, conventional Pap stain showed better staining results as compared to REAP but statistically not significant (>0.05). On conventional Pap stain 398 cases out of 480 cases had distinct cytoplasmic borders, 104 had excellent, 284 had satisfactory and 92 were unsatisfactory for cytoplasmic staining. 379 cases had distinct nuclear borders and 361 had crisp chromatin staining. On REAP staining, 382 cases had distinct cytoplasmic borders, 102 had excellent, 280 satisfactory and 98 were unsatisfactory for cytoplasmic staining. 363 cases had distinct nuclear borders and 352 had crisp chromatin staining.

On REAP staining, 41 cases were diagnosed with bacterial vaginosis, 8 cases with candidiasis and 2 cases with trichomonal vaginitis. On conventional stain, 41 cases had bacterial vaginosis, 9 cases with candidiasis and 3 cases with trichomonal vaginitis. These results were comparable, showing the diagnostic efficacy was comparable and not compromised as the conventional technique is the gold standard for Pap smear staining.

Deshpande et al[10] concluded in his study that REAP showed better staining of candida and trichomonas vaginalis with clear background without any debris as compared to conventional Pap stain. In our study although conventional had better staining results for both organisms. In this study out of 500 cases, 21 cases and 22 cases had squamous epithelial cell abnormality on conventional and REAP stain respectively.Results were similar, except on conventional stain, 6 cases were diagnosed as ASCUS whereas 7 cases on REAP stain. ASC-H, LSIL, HSIL and SCC were 4, 4, 5 and 2 respectively on both conventional and REAP stain. The results were almost similar and statistically insignificant.

The results were accordingly compared with other studies for Bethesda System for Cervical Cytology.

Gupta et al[26] concluded that out of 480 smears, 14 (2.9%) showed epithelial cell abnormality out of which 7 cases were of ASCUS on conventional staining and other 466 (97.1%) were reported as NILM. 6 (1.2%) caseswere found with glandular cell abnormality out of which 4 (0.8%) cases were of atypical glandular cell, NOS and 2 (0.4%) were of atypical endocervical cell on both conventional and REAP stain.The result was similar and statistically insignificant.

In this study for rapid staining of Pap smear, REAP technique was used in which total duration was 7 minutes whereas the staining duration for conventional stain was 3 times of REAP.

Biswas et al[10] studied REAP, the turnaround time was 3 minutes only for REAP and 20 minutes for conventional stain.

In study by Gachie et al[22], the turnaround time was same whereas in study by Deshpande et al[19] the time for REAP was 7 minutes but for conventional was 35 minutes.

Izhar et al[23] concluded that REAP reduced the staining time but cannot be used for routine staining of Pap smear due to poor preservation of staining quality.

In our study the smear preservation after 1 year was good with both conventional and REAP techniques. As the ethyl acetate act as preservative for cytoplasmic staining and fixative for nuclear stain whereas on conventional the staining was not long standing.

Both Dighe et al[16] and Deshpande et al[19] concluded that both conventional and REAP showed the excellent smear preservation for 2 years.

Both Biswas et al[15] and Hussein et al[15] observed excellent smear preservation till 6 months.

Gachie et al[27] did not include this parameter in their study. In this study, the cost of staining for single Pap smear was difficult to calculate but there is less consumption of alcohol in REAP as compared to conventional stain.

Alcohol was used only for fixation of Pap smear and in the final step where methanol was used before final dehydration in xylene. In all other steps, acetic acid was used, only 10 dips for each step.

Vani et al[36] and Biswas et al[11] included this parameter in their studies and the result showed the cost of conventional stain per slide was 4 times the cost of REAP.

Gachie et al[27] concluded that the cost for conventional was almost 6 times the cost for REAP stain whereas in study done by Dighe et al[21] the cost was 8 times of cost of REAP.

It was concluded that the cost for REAP is less than the conventional. Acetic acid is easily available and cheaper than alcohol.

In this rapid staining technique of Pap smear, the cellular details are improved with better cytomorphological features. The satisfactory and unsatisfactory smears were same because of same technique of sample collection but the cytoplasmic and nuclear features were better visualised with REAP as compared to conventional stain.

The nuclear border and the chromatin details are crisp with REAP stain with distinct cytoplasmic borders and its staining. Without compromising the diagnostic efficacy REAP technique is 3 times faster and easier than the conventional stain thus easier for the technicians to learn. Amount of alcohol used is less in REAP so obviously cost effective also.

CONCLUSION

In conclusion, REAP is a simple, easy and cheaper staining method with good cytomorphological features over conventional staining. There is no compromise in the diagnostic efficacy although conventional is the gold standard method for Pap smear staining.

REAP has a very simple and rapid technique with good smear preservation, can easily be followed in resource limited areas. Hence in view of all the advantages, it would be worthwhile to use this technique for rapid staining result.

Table 1: Specimen Adequacy

	Smear staining (n=500)	
	No. of Cases	%
Satisfactory for evaluation	383	76.6
Satisfactory for evaluation but limited by 1. Partially obscured by blood 2. Partially obscured by inflammation	96	19.2

Unsatisfactory for evaluation because of 1. Low cellularity 2. Obscuring blood 3. Obscuring inflammation 4. Air drying artefacts	21	4.2

Table 2: Comparison of cytomorphological features on Conventional stain and REAP

Parameters	Conventional		REAP		P-value	
	Cases	%	Cases	%		
Cell/Cytoplasmic borders						
Distinct	464	92.8	472	94.4	0.01259	S
Indistinct	36	7.2	28	5.6		
Cytoplasmic staining						
Satisfactory	428	85.6	448	89.6	0.00569	S
Unsatisfactory	72	14.4	52	10.4		
Nuclear border						
Distinct	464	92.8	471	94.2	0.03720	S
Indistinct	36	7.2	29	5.8		
Chromatin staining						
Distinct	465	93	473	94.6	0.02765	S
Hazy	35	7	27	5.4		

Table 3: Comparison of cytoplasmic stain, nuclear stain, smear preservation, turnaround time on Conventional stains and REAP.

Staining Technique	Cytoplasmic stain Optimal		Nuclear stain Optimal		Staining time (minutes)	Smear preservation for 1 year
	Cases	%	Cases	%		
Conventional Stain	428	85.6	441	88.2	20	Good
REAP	448	89.6	466	93.2	7	Good

Table 4: Distribution of Type of Lesions on Conventional stain and REAP

Lesions	Conventional stain (n=500)		REAP (n=500)	
	No. of cases	%	No. of cases	%
NILM	452	90.4	451	90.2
Epithelial cell abnormality	21	4.2	22	4.4
Glandular cell abnormality	6	1.2	6	1.2
Unsatisfactory	21	4.2	21	4.2
Total	500	100	500	100

Table 5: Interpretation and comparison of NILM category on Conventional stain and REAP

Parameters	Conventional Stain		REAP	
	No. of Cases	%	No. of Cases	%
Inflammatory/ Reactive	305	61	306	61.2
NILM	54	10.8	54	10.8
Atrophic smear	40	8	40	8
Bacterial vaginosis	41	8.2	41	8.2
Candidiasis	09	1.8	08	1.6
Trichomonal vaginitis	03	0.6	02	0.4
Total	452	90.4	451	90.2

Table 6: Interpretation and comparison of Epithelial cell abnormality on Conventional stain and REAP

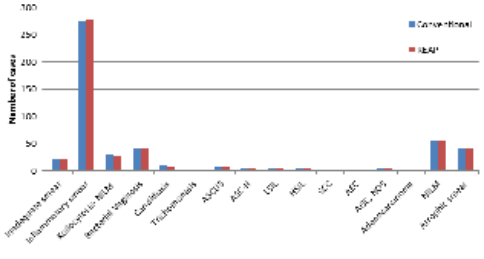
Epithelial cell abnormality	Conventional Stain (n=500)		REAP (n=500)	
	No. of cases	%	No. of cases	%
ASCUS	6	1.2	7	1.4
ASC-H	4	0.8	4	0.8
LSIL	4	0.8	4	0.8
HSIL	5	1	5	1
SCC	2	0.4	2	0.4
TOTAL	21	4.2	22	4.4

Table 7: Interpretation and comparison of Glandular cell abnormality on Conventional stain and REAP

Glandular cell abnormality	Conventional Stain (n=500)		REAP (n=500)	
	No. of cases	%	No. of cases	%
Atypical Glandular Cells, Not Otherwise Specified	04	0.8	04	0.8
Atypical Endocervical Cells	02	0.2	02	0.2
Adenocarcinoma	00	00	00	00

Table 8: Comparison of Diagnosis

Diagnosis	Conventional Stain		REAP		p-value	
	No. of cases (n=500)	%	No. of cases (n=500)	%		
Inadequate smear	21	4.2	21	4.2	1.00000	NS
Inflammatory smear-NILM	276	55.2	278	55.6	0.93228	NS
Inflammatory Smear with koilocytosis-NILM	29	5.8	28	5.6	0.89462	NS
Bacterial Vaginosis – NILM	41	8.2	41	8.2	1.00000	NS
Candidiasis – NILM	09	1.8	08	1.6	0.80836	NS
Trichomonal Vaginitis – NILM	03	0.6	02	0.4	0.65472	NS
ASCUS	06	1.2	07	1.4	0.78151	NS
ASC-H	04	0.8	04	0.8	1.00000	NS
LSIL	04	0.8	04	0.8	1.00000	NS
HSIL	05	1	05	1	1.00000	NS
SCC	02	0.4	02	0.4	1.00000	NS
Atypical Endocervical cells	02	0.4	02	0.4	1.00000	NS
Atypical glandular cell, NOS	04	0.8	04	0.8	1.00000	NS
Adenocarcinoma	00	00	00	00	00	NS
NILM	54	10.8	54	10.8	1.00000	NS
Atrophic smear	40	8	40	8	1.00000	NS



Graph 1-- showing comparison of diagnosis between Convention and REAP

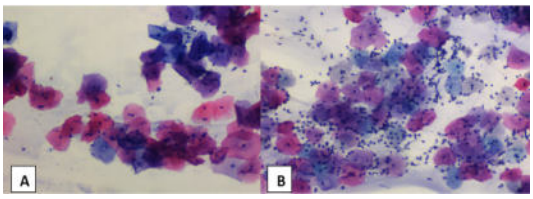


Figure 1-A) Conventional Pap stain (Pap Stain, 100x), B) REAP stain (Pap Stain, 100x)

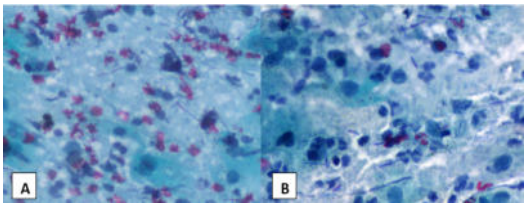


Figure 2- A) Candidiasis on conventional stain (Pap stain, 400x), B)- Candidiasis on REAP stain (Pap stain, 400x)

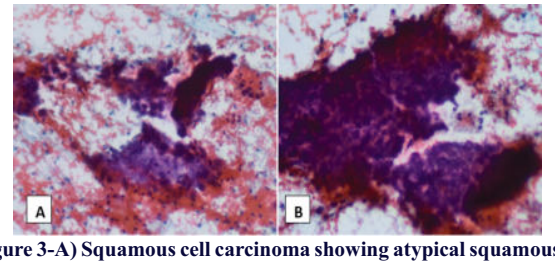


Figure 3-A) Squamous cell carcinoma showing atypical squamous cells with high N:C ratio and tumour diathesis on conventional stain (Pap stain 100x), B) Squamous cell carcinoma showing atypical squamous cells with high N:C ratio and tumour diathesis on REAP stain (Pap stain 100x)

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