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Original Research Article

Evaluation of efficacy of an eco friendly enviropap and REAP stain in cervical cytology at resource limited area

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ABSTRACT

Background: Cervical cancer is integral to any cancer control program in India because of the disease burden. Quick diagnosis plays a crucial role in limited resource areas. Hence, cost-effective and eco-friendly staining procedures with less time consumption help screening for cervical cancer cytodiagnosis. Thus, we wanted to assess the superiority of staining of smears by REAP (Rapid Economical Acetic acid Papanicolaou) technique and the newer EnviroPap (Environmental Papanicolaou) technique compared to conventional Pap technique on cervical smears.
Methods: From each patient, three smears were prepared, one with routine conventional method, one with REAP stain, and another with EnviroPap stain and quality parameters were noted. The chi-square test assessed differences in categorical variables between stains; a p value of <0.05 was considered significant. Statistical analysis used was the Chi-square test, and p value of <0.05 using a two-tailed test was taken as being significant for all statistical tests.
Results: REAP and Enviropap stained smears showed excellent nuclear and cytoplasmic details compared to conventional Pap staining protocol.
Conclusions: REAP and EnviroPap staining protocols for those two techniques were simpler, easier, and cost-effective, which took minimal turnaround time for assessing pap smears at a large scale.

Keywords: Cytological technique, Cancer screening, Enviro pap, Pap smear, REAP

INTRODUCTION

Cervical cancer is the most common cancer in women in developing countries like India, where approximately 1,22,844 women are diagnosed and 67,477 deaths annually. Cervical cancer is essential to any cancer control program in India because of the disease burden.[1]

Prevention efforts worldwide have concentrated on screening programs for women at risk of the disease using Pap smears and treating as early as possible.[1-2]

The screening program should ensure broad coverage of the target population and effective onsite results so that immediate treatment, if abnormal, can be taken.[1] Poverty, limited access of the people to information, and lack of healthcare infrastructure are some of the drawbacks of the implementation of cervical cancer screening programs. Due to little or low-quality cytological services, diagnostic and treatment services have proven obstacles in screening camps to be effective.[1]

Pap (Papanicoloau) staining was first introduced by Papanicoloau in 1943 and is a reliable technique used for cervical screening worldwide.[3] The original Pap staining protocol is time-consuming and requires a large amount of alcohol; because of these drawbacks, it has undergone various modifications to decrease turnaround time or find

an alternative to alcohol to be cost-effective without compromising the quality or the cytodiagnosis of the smears.[4-6]

Quick diagnosis plays a crucial role in cervical cancer screening programs in limited resource areas and is gaining popularity due to the global trend in reducing health care costs.[1] Hence cost cost-effective and environmentally friendly staining procedures are helpful in screening for cervical cancer cytodiagnosis.[7-8] EnviroPap stains use a specified group of products and process that yields high-quality, reproducible staining results, which save money, bypassing those chemicals defined for bluing agents, consuming less alcohol, reusing xylene indefinitely, and reducing hazardous waste disposal costs.[7]

Rapid, Economic, Acetic Acid Papanicolaou Stain (REAP) technique modified PAP staining, which can be an alternative to conventional PAP procedure. A few cytotechnologists observe some drawbacks, like poor penetration properties and suboptimal staining quality.[2,4]

Thus, this study to assess the quality of staining of smears by REAP technique and the newer EnviroPap technique when compared to conventional Pap technique on cervical smears based on the following parameters: a) Intensity of nuclear and cytoplasmic staining, b) overall staining quality, c) time taken for staining, and d) cost-effectiveness.

METHODS

Ours is a prospective study (July 2021 to August 2022) done where 200 samples were collected from the Department of Obstetrics and Gynecology (OBG) Outpatient Section, and staining was done at the cytology section, Department of Pathology in Sri Devaraj Urs Medical College. Institutional ethical consent was obtained before the start of the study.

Inclusion criteria

All patients with any gynecological problems like white discharge, bleeding history, mass per vagina, irregular growth, polyps, bleeds on touch, and included irregular menstrual history visiting for cervical cancer screening program at the Department of Obstetrics and Gynecology in our study after taking informed consent.

Exclusion criteria

Excluded vault smears since no endocervical cells will be seen; comparison not possible.

From each patient, three smears were prepared and fixed with bio spray fixatives and transported to the pathology department's cytology section. Each slide was then stained, one with the routine Conventional method, one with REAP stain, and another with EnviroPap stain. conventional method and REAP, we used commercially available by BioLab diagnostics papanicolaou stain kit and AMD labs, respectively. EA 30 dye taken for EnviroPap bought from AMD Labs Company. A routine staining procedure was used to stain the smears for conventional Pap stain (Table 1).

Table 1: Procedure of reap and enviropap staining.

REAP stain method	EnviroPap stain method
Smears fixed in alcohol	Tap water-10 dips
1% acetic acid -10dips	Tap water-10 dips
Harris hematoxylin-10 dips	Harris Hematoxylin -1 to 2mins
Tap water-10 dips	Tap water-10 dips
1% acetic acid -10 dips	Tap water-10 dips
OG-6-10 dips	Tap water-2mins
1% acetic acid-10 dips	Tap water-10 dips
EA 50- 10 dips	OG 6- 10 secs
1% acetic acid-10 dips	0.5% Acetic acid- 10 dips
Methanol-10 dips	0.5 % Acetic acid-10 dips
Xylene-10 dips	0.5 % Acetic acid-10 dips
Mount with coverslip Staining technique total time taken: 5 minutes	EA 50- 8 mins
	0.5% Acetic acid-10 dips
	0.5% Acetic acid-10 dips
	0.5 % acetic acid-10 dips
	Absolute ethanol-10 dips
	Absolute ethanol-10 dips
	Absolute ethanol-10 dips
	Xylene- 10 dips
	Xylene- 10 dips
	Xylene- 10 dips Staining technique total time taken: 10 minutes

We did a few modifications in REAP compared to conventional PAP are as follows: a) Ethanol bath in pre-OG6, post OG6 and post EA36 stages is replaced by acetic acid concentration 1%, b) Tap water is replaced with Scotts tap water, c) For rapid penetration of haematoxylin-preheated in water bath to 60^{o}C, d) For final dehydration, only methanol is used.[8,9,10]

In enviropap compared to conventional PAP are as follows: A) Water is used to replace 95% ethanol to remove carbowax from spray-fixed slides, B) No graded alcohols, C) For blueing hematoxylin, plain tap water is used, D) Plain tap water before OG, and E) 0.5% acetic acid solutions are replacing 95% ethanol following OG and EA.[7]

Enviro-Pap eliminates a) Frequent usage of 95% ethanol baths, b) “Chemically bought” bluing agents, and, c) Xylene disposal reused the same using Silicone gel beads.[7]

The original labels of the stained smears were replaced by another set of identification codes unknown to two

pathologists screening the slides to avoid bias and screening the cases for cytomorphological parameters to assess the staining qualities. Then, these stained smears were examined by two pathologists, and the following parameters were assessed (Table 2).

Table 2: Parameters of stained smears were examined by two pathologists.

	Score
Background	
Hemorrhagic	1
Clean	2
Cell morphology	
Not preserved	1
Moderately preserved	2
Well preserved	3
Nuclear characteristics	
Dull	1
Moderately crisp	2
Crisp	3
Overall staining	
Bad	1
Moderately good	2
Good	3
Air drying artifacts	
>50%	1
<50%	2
0%	3
Cytoplasmic details	
Unsatisfactory	1
Suboptimal	2
Optimal	3
Inflammation	
Dense	1
Moderate	2
Minimal	3
Clean	4
Organisms	
Present	1
Absent	2

The smears were then numerically evaluated and given scores. The maximum score from all eight parameters was 23. Quality index (QI) was calculated using:

QI=Actual score obtained/Max score

The QI for the three stains was calculated, compared, and analyzed. Mean quality index for each stain was calculated and analyzed. A common consensus was arrived at in the cases where there was a difference in the scores.

Statistical analysis

The data are reported as the mean +/- SD or the median, depending on their distribution. Frequencies were expressed in percentages. The chi-square test was used to assess differences in categorical variables between stains. Took *a* p-value of <0.05 using a two-tailed test as being of significance for all statistical tests. Analyzed all data using a statistical software package- SPSS version 22 (IBM, Corporation).

RESULTS

A total of 200 patients samples were collected, and three smears stained by conventional Papanicolaou, REAP stain, and EnviroPap were examined. The minimum age of the patients screened was 27 years, and the maximum was 65 years. The majority were in the age group of 31-40 years. The mean age was 37 years (Table 3).

Table 3: Demographic data of the patients.

Age of the patients (years)	Total number of the patients	Percentage
20-30	24	12
31 40	96	48
41-50	38	19
51-60	25	12.5
61-70	17	8.5

Most common clinical features patients presented with white discharge per vagina 39% (78/200 cases, followed by menorrhagia 33% (65/200 cases), irregular bleeding 14% (28/200 cases) and routine 14 % (28/200 cases).

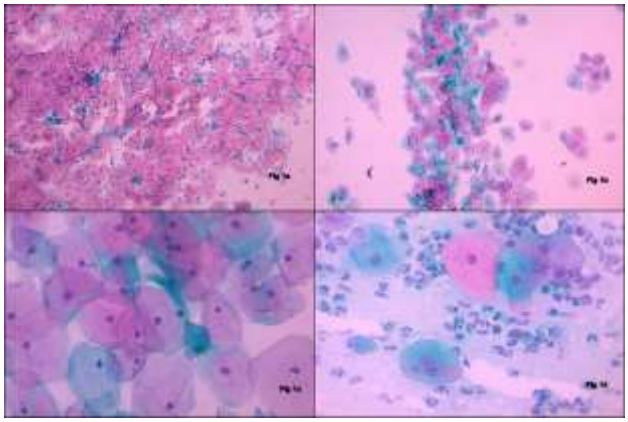

Figure 1: REAP stain. a) Celluarity of the smear (at low magnification), b) Clusters of epithelial cells (at 20X magnification), c) Quality of staining patterns (at 40 X magnifications), d) Background (at 40X magnifications).

Most common epithelial abnormalities were Negative for Intraepithelial Lesion or Malignancy (NILM) 74.5% (149/200 cases), low-grade squamous intraepithelial lesion (LSIL) 12% (24/200 cases). Squamous cell carcinoma 6% (12/200 cases), high grade squamous intraepithelial lesion (HSIL) 3% (6/200 cases), atypical glandular cells of undetermined significance 1% (2 /200 cases) and inadequate sample 3.5% (7/200 cases). All the

cases findings and diagnoses of the smears stained by REAP and EnviroPap techniques correlated with their corresponding smears stained by conventional Pap staining technique. The staining reactions for non-epithelial cells like red blood cells and white blood cells and staining of bacteria showed no changes and were similar in all three stains.

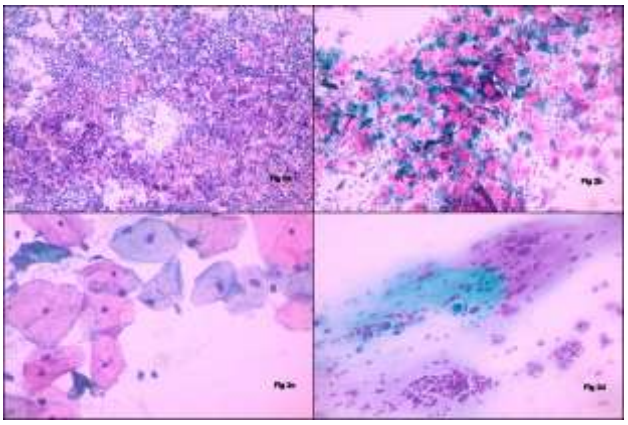

Figure 2: EnviroPap stain. a) Celluarity of the smear (at low magnification), b) Clusters of cells (at 20X magnification), c) Quality of staining patterns (at 40 X magnifications), d) Background (at 40X magnifications).

The cytoplasmic differentiation was optimal in REAP, and EnviroPap was 68% and 79%, respectively. In 32% and 21%, the cytoplasmic stain penetration was suboptimal in thickness and overlapping clusters in REAP and EnviroPap staining techniques, respectively. The nuclear details and chromatin pattern were clear and crisp was 75% and 84% in REAP and EnviroPap staining, respectively. The overall staining was good in REAP, and EnviroPap was 75% and 80%, respectively. Drying artifacts was very low in these staining techniques. The mean quality index of REAP and EnviroPap were correlated with conventional pap stain.

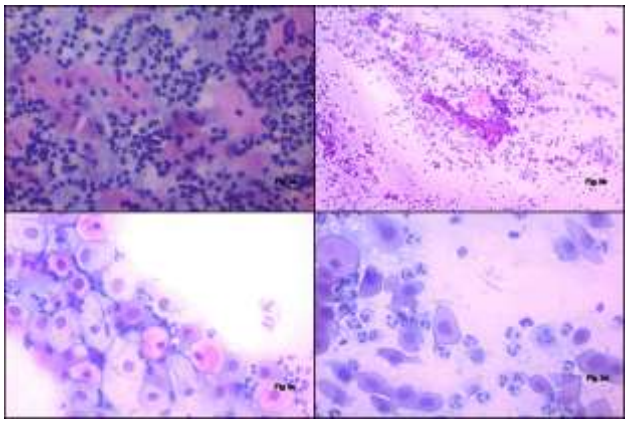

Figure 3: Routine conventional Pap stain. a) Celluarity of the smear (at low magnification), b) Clusters of epithelial cells (at 20X magnification), c) Quality of staining patterns (at 40 X magnifications), d) Background (at 40X magnifications).

The turnaround time for REAP was 5mins and EnviroPap stain 10mins compared to conventional pap stain 20mins. The cost per slide for REAP stain and EnviroPap staining was much less when compared to conventional pap stain. Since the cost of acetic acid costs was approximately 1/6th of the total price of absolute alcohol.

REAP (Figure 1) and EnviroPap (Figure 2) stained smears were compared with conventional PAP (Figure 3) stained smears.

Compared to conventional Pap staining protocol, REAP and Enviropap stained smears showed excellent nuclear and cytoplasmic details (Table 4).

Table 4: Associations of quality parameters with conventional Pap, REAP and EnviroPap stains.

Quality parameters	Conventional Pap %	REAP %	EnviroPap %	Chi-square p-value
Cell morphology				
Not Preserved	18	9	0	32.27
Moderately Preserved	46	37	30	
Well preserved	36	54	70	0.000001688
Nuclear characteristics				
Dull	19	5	0	76.25
Moderately crisp	52	20	16	
Crisp	29	75	84	<0.0000001
Overall staining				
Bad	13	0	0	50.65
Moderately good	45	25	20	
Good	42	75	80	<0.0000001
Air drying artifacts				
>50%	14	0	0	42.49
<50%	52	46	39	
0%	34	54	71	<0.0000001

Continued.

Quality parameters	Conventional Pap %	REAP %	EnviroPap %	Chi-square p-value
Cytoplasmic details				
Unsatisfactory	15	0	0	71.17
Suboptimal	56	32	21	
Optimal	29	68	79	<0.0000001
Mean quality index	0.72	0.81	0.84	

DISCUSSION

Cervical cancer is seen as the most common cancer in women in India. For the past 30 years, in India, regular screening programs have been conducted for which only little impact has resulted. Mainly because screening camps are available only to a small proportion of women through private health care providers or the public sector to women not in the high-risk groups.[1] Staining techniques to be utilized in mass screening programs should have good characteristics; techniques must be easy, rapid, eco-friendly, and economical with minimal turnaround time.[1,5,8]

The most reliable technique for cervical cancer screening programs is conventional Papanicolaou stain staining, commonly employed in India.[5] There are certain limitations: ethanol is used enormously as a dehydrating agent, which is costly, requires a purchase license, and is a time-consuming procedure.[2-6] Another probable cause that is not justified yet due to the lack of scientific documenting evidence of the tremendous negative ecological impact caused by the daily massive usage and disposal of reagents for this routine Pap, such as:

A. hydrochloric acid- which should change at least once daily.[11-13]

B. Alcohol is used in large amounts as a rehydrating and dehydrating agent before the cytoplasmic stains. It should be checked occasionally with a hydrometer to avoid troubleshooting; it should replaced weekly or may be discarded each day to prevent the necessity of filtering these solutions.[12-14]

C. Xylene is usually used for clarifying the cells' cytoplasm. We should change xylene when it appears tinted with any cytoplasmic stains. Water in the xylene will make the solution appear slightly milky. The clearing process may be disturbed, and tiny drops of water can be seen microscopically on a plane above the cell on a slide.[12-15]

Thus, to fulfill the criteria, developed a series of modifications for PAP staining for faster impact on the treatment aspect for better patient care.

REAP technique is the latest modification of the standard PAP technique. It stands for rapid economic acetic acid Papanicoaous stain.[4-6] As the name implies, this technique is the fastest, and cost effective; acetic acid is used as a dehydrator and color preservation. These features have made this technique superior to that of routine conventional PAP. This finding was proven by Asthana et al, Vani et al and Biswas et al in their studies.[4-6]

We prepared REAP stain by replacing alcohol with 1% acetic acid except for the first step of fixation and the last step of dehydration, where absolute alcohol as in routine Pap stain. Thus, acetic acid acts as a mild dehydrating agent, nuclear fixative, increases the staining intensity of the nucleus, preserves the color of the stain, makes rapid staining, and is cheap and easily available (Asthana A, 2014; Izhar et al, 2014; Biswas et al, 2008).[2,4,16] For ensuring faster penetration of dye particles into cells, the Harris Hematoxylin is preheated to 60C. The slides were blotted after each step, reducing the reagent contamination issues. We did not do any acid differentiation. Blueing uses less time in ordinary tap water than Scott's tap water. The time for cytoplasmic stain is reduced, and concentration is four times more. Methanol is used for final dehydration.[4] OG-6 and EA50 are alcohol-based stains. In routine Pap stain, after cytoplasmic staining, alcohol diffuses into a dehydrating medium (ethanol), resulting in decreased staining intensity. In REAP stain, 1% acetic acid reacts with ethanol of cytoplasmic stain and results in ethyl acetate and water. Will remove from the cell. The ester (ethyl acetate) complexes with cytoplasmic stain are deposited in the cells, preserving staining intensity. In routine pap stain following dehydration with ethanol, some ethanol enters and remains in the cell after clearing. This ethanol follows mounting with DPX and sometimes later dissolves cytoplasmic and nuclear stain, which percolates into DPX. Hence, color preservation does not last long. In REAP, ethyl acetate preserves cytoplasmic staining, and acetic acid, a nuclear fixative, preserves nuclear stain.[2,4,16]

The cytoplasmic transparency and nuclear details are statistically superior in the REAP stained smears compared to the smears stained by routine PAP in our study supported by other studies Biswas et al, Dighe et al, Gachie et al.[16-18] An insight into the chemistry of the staining techniques reveals a probable explanation for the better results obtained by REAP over PAP.

The quality of REAP staining was as good as conventional PAP, whereas considerably reduced turnaround time and cost per smear in REAP.[16-18]

a. Quality of staining - Biswas et al, Gachie et al, REAP fared better than conventional PAP as in Dighe et al and present study REAP fared slightly better than conventional PAP.[16-18]

b. Dighe et al, Biswas et al, and Gachie et al, the time consumed for conventional PAP and REAP was 20 minutes and 3 minutes, respectively, and reduced the cost per slide for REAP by 4 to 6 times.[16-18]

c. In the present study, the time consumed for PAP and REAP was 30 and 7 minutes, respectively. We have reduced the cost per slide by four times.

Enviro-Pap is an eco-friendly, cost-effective modification which developed in 1995. It is a group of ingredients and a quality process that yields highly reproducible staining results while saving money by replacing chemically defined bluing agents (e.g., Scott's tap water substitute), consuming less alcohol, reusing xylene indefinitely, and reducing hazardous waste disposal costs.[7]

Papanicolaou experimented and formulated 3 times between 1942 and 1960 where he significantly changed the OG and EA dye formulations, but he never gave standard compositions in quantitatively reproducible terms.[7]

Consequently, all cytotechnology programs teach a different Pap stain, all vendors sell different formulations, and all laboratories have their own staining protocols.[18-20]

Since commercially available OG and EA solutions are so variable in composition, we can only use the recommended staining times in OG and EA for Enviro-Pap with some initial experimentation on the part of the laboratory users. We used buccal smears as quality assurance to confirm the satisfactory performance of the individual stains as an indicator. [7] Following the general rule: “less time in OG and more time in EA”: we concluded that in OG-10 secs and EA-8 mins was finalized after repeated experiments and used Silica-gel pellets in xylene, minimizing the possibility of water contamination and absorption which will give long life for xylene and thus can reused with less disposal of xylene.[7,20]

The staining qualities of both nuclear and cytoplasmic details in REAP and Enviropap were better than conventional Pap, but the diagnosis in the three methods was the same. Similar findings were observed in Asthana et al, Dighe et al, Biswas et al, Gachie et al studies for REAP.[4,16-18] Irrespective of different clinical presentations, both REAP and EnviroPap stains showed better background and cell morphology when compared to conventional Pap. Our study revealed that lysis of red blood cells in the hemorrhagic smears with no change in squamous cell morphology due to acetic acid in both Enviropap and REAP stain facilitated the interpretation of cervical smears (Table 5).

Table 5: Comparison of quality of stains with other studies.

Authors	Stains	Cytoplasm stain (%)		Nuclear stain (%)	
		Sub optimal	Optimal	Sub optimal	Optimal
Dighe et al, (n=200) 2006	Pap	-	200 (100)	-	200 (100)
	REAP	19 (9.5)	181 (90.5)	8 (4)	192 (96)
Biswas et al, (n=110) 2008	Pap	20 (18.2)	90 (81.8)	10 (9.1)	100 (90.9)
	REAP	10 (9.1)	100 (90.9)	5 (4.6)	105 (95.4)
Gachie et al, (n=159) 2011	Pap	29 (18.3)	130 (81.7)	29 (18.3)	130 (81.7)
	REAP	21 (13.3)	138 (86.7)	21 (13.3)	138 (86.7)
Vani et al, (n=100) 2017	Pap	2 (2)	98 (98)	2 (2)	98 (98)
	REAP	4 (4)	96 (96)	4 (4)	96 (96)
Present study, (n=80) 2019	Pap	30 (15)	170 (85)	38 (19)	162 (81)
	REAP	-	200 (100)	10 (5)	190 (95)
	EnviroPap	-	200 (100)	-	200 (100)

Additionally, we also observed that the morphology of the endocervical cells, when present singly, were better stained than when in clusters in Enviropap and REAP, which could be attributed to poor penetration of the stain in overlapping clusters of cells.

This study has some limitations. Newer stains have suboptimal staining for thicker cervical smears, which needs slight modifications in the steps to overcome. We could not assess the shelf-life of the stained smears, like how long the stained smears can be preserved. Rapid diagnosis in mass screening camps will be helpful, but using the resources for research is challenging.

CONCLUSION

Thus, we conclude that these modified stains can be used in mass screening camps in resource-limited areas since it has feasible staining techniques. Staining protocols for those two techniques was more straightforward, easier, and cost-effective, which took minimal turnaround time for assessing pap smears at a large scale. The costs

involved in REAP and EnviroPap methods were lesser when compared to the PAP technique. The REAP method took 7 minutes, the EnviroPap method took 12 minutes, whereas the conventional PAP method involved 30 minutes.

Compared with conventional Pap, good cytoplasmic transparency, optimal nuclear details, and transparent background were seen in REAP and EnviroPap stained smears.

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