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Research paper

Effectiveness of modified Papanicolaou staining method used in a Sri Lankan government sector laboratory: a cost-effective method for resource limited settings

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Abstract

Background and Objective: The study investigates the effectiveness of a modified Papanicolaou staining method for cervical screening, currently practiced at District General Hospital Matara (DGHM), Sri Lanka, as the conventional method requires a high amount of alcohol.

Materials and Methods: A cross-sectional study was conducted from September to November 2023 in the histopathology laboratory at DGHM. A total of 161 cervical smears were collected from Medical Officer of Health (MOH) areas in Matara. Paired smears were stained using both the conventional and modified Pap methods. The modified method involved replacing the alcohol series for hydration with 70% alcohol. After staining with Harris' haematoxylin and rinsing with tap water, the differentiation step in 0.5% HCl and dehydration step with an alcohol series were omitted. Tap water replaced the use of 95% alcohol for rinsing after staining with OG6 and EA50. Smears were air-dried, mounted, and blindly evaluated by the primary investigator, two cyto screeners, and a consultant histopathologist using a standardized scoring system. A quality index was calculated for each method.

Results: Out of 152 satisfactory paired smears, 98.7% of conventionally stained and 96.7% of modified-method smears showed distinct cytoplasmic borders. Satisfactory cytoplasmic staining was observed in 60.5% and 57.2% of conventional and modified smears, respectively. All smears showed distinct nuclear borders and crisp chromatin staining. There was no statistically significant difference in cytomorphological quality ($p > 0.05$). The quality index for the modified and conventional methods was 0.911 and 0.932, respectively. The modified method reduced alcohol use by 80%, resulting in significant cost savings.

Conclusion: The modified Pap staining method is a cost-effective, simpler alternative suitable for cervical cytology in resource-limited settings without compromising diagnostic quality.

Keywords: cervical screening, Papanicolaou smear, modified Papanicolaou staining

Introduction

Invasive carcinoma of the cervix is one of the most common cancers in the world. In Sri Lanka, it is the second most common cancer among women(1,2,3). Cervical cancer is highly preventable and curable if detected early.

Cytological screening can detect early cervical cancer precursors, including atypical squamous cells of undetermined significance (ASCUS), low-grade squamous intraepithelial lesions (LSIL), high-grade squamous intraepithelial lesions (HSIL), and atypical

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glandular cells(4). Screening reduces mortality by 70% and the probability of developing invasive carcinoma is decreased by over 95%(5).

The universal stain for cervical cytological screening is the Papanicolaou stain, which yields a polychromatic transparent staining reaction(6,7). Conventional Papanicolaou stain is known for its good cytohistological correlation(8-11). Since this screening method was introduced by George Papanicolaou in 1943(8), it has undergone various modifications since this standard method consumes a substantial quantity of alcohol, which is expensive and difficult to procure. Further, the procedure takes time (more than 20 minutes) and requires Xylene, which is carcinogenic(12). The Papanicolaou stain has been modified as Ultra-Fast Papanicolaou stain (UFP) and Modified Ultra-Fast Papanicolaou stain (MUFP). Both UFP and MUFP used air-dried cervical smears, and staining time was reduced(13,14). In addition, Rapid-Economic-Acetic Acid-Papanicolaou stain(REAP) was developed where alcohol is replaced by 1% acetic acid. Studies conducted by Dighe et al. and Biswas et al., showed that the REAP method was an excellent and rapid alternative for cytological screening with minimum cost(15-17).

District General Hospital, Matara (DGHM), Sri Lanka uses a modified Papanicolaou staining method that requires less alcohol and time and requires no xylene. The conventional Papanicolaou smear is cheaper than other methods used for staining cervical smears, since it requires less alcohol. Evaluating the effectiveness of this modified method in comparison to the conventional Papanicolaou technique, and introducing it into the healthcare system, could be highly beneficial for a developing country like Sri Lanka; particularly in the context of the current financial crisis. Accordingly, the primary objective of this study was to assess the effectiveness of the modified Papanicolaou staining method in detecting precursors of cervical carcinoma.

Methods

A cross sectional study was conducted during a period of two months at DGHM. One additional cervical smear was prepared from the consented women aged between 35 and

Table 1: Steps of standard (conventional) and modified Papanicolaou methods

Conventional Papanicolaou method	Modified Papanicolaou method
1.Hydrate in 95% alcohol – 2 min	Hydrate in 70% alcohol- 2 min
2.Hydrate in 70% alcohol – 2 min	
3.Rinse in water	
4.Stain in Harris's haematoxylin- 5 min	Stain in Harris's haematoxylin- 1 min
5.Rinse in tap water – 2 min	Rinse in tap water – 2 min
6.Differentiate in 0.5% aqueous hydrochloric acid- 10 secs **7.Rinse in water – 2 min** **8.Blue in Scott's tap water- 2 min** **9.Rinse in water – 2 min. Dehydrate in 70% alcohol- 2 min** **10.Dehydrate in 95% alcohol- 2 min** **11.Dehydrate in 95% alcohol- 2 min**	
12.Stain in OG6- 2 min	Stain in OG6 – 2 min
13.Rinse in 95% alcohol- 2 min	
14.Rinse in 95% alcohol – 2 min	Rinse in tap water 30 secs
15.Stain in EA50- 3 mins	Stain EA50 – 2 mins
16.Rinse in 95% alcohol – 1 min	
17.Add in xylene – 2 min	Rinse in tap water- 1 min
18.Add in xylene – 2 min	
19.Air dry and mount with DPX	Air dry and mount with DPX

55 years who attended the Well Women clinics of their medical officer of health (MOH) areas for cervical screening. Cervical smears were prepared by the medical officers/ field nurses in selected MOH areas. Both smears prepared from the same individual were labelled with a specific serial number. The smears were immediately fixed in 95% alcohol for 30 minutes. After transporting them to the histopathology laboratory of DGHM, one slide was stained with modified Papanicolaou staining method and the other one was stained with standard Papanicolaou method. TISSUE-TEC DRS-2000 automatic slide stainer machine was used for both the procedures. Slides were selected randomly for the two staining procedures (Table 1).

After mounting with DPX, slides were labelled properly. Smears which were satisfactory to screen were screened by the primary investigator who was blinded to the staining protocol. Smears containing approximately 8000-12000 well visualized squamous cells were included in the study. Smears with an estimated squamous cell count of less than 8000(18), highly inflammatory, poorly preserved and air-dried smears were excluded from the study.

Results were entered and evaluated according to the Bethesda system 2001 (19,20). The various nuclear and cytoplasmic parameters for staining quality and morphological details were assessed, scored and recorded according to the scoring system (Table 2) which was defined by Gachie et al(5).

According to the inclusion and exclusion criteria, nine out of the 161 pairs of smears collected, were found to be unsatisfactory. A total of 152 paired smears were evaluated further and were re-screened by two cyto-screeners and the consultant histopathologist of DGHM who were blinded to the staining protocol. After completion of the reporting and scoring, the staining methods of the slides were revealed and the slides were decoded and assigned to the respective methods separately. Each pair of slides was compared separately.

The evaluation of the two Papanicolaou staining methods by cytomorphological features, was done by means of Quality Index, introduced by Chan, et al. 1988(21).

$$\text{Quality Index} = \frac{\text{Total actual score obtained}}{\text{Maximum score}}$$

Total actual score was calculated by adding the score obtained by each subject for each parameter (21).

Statistical analysis

The reporting was done using the Bethesda System of 2001 for reporting of cervical smears. The percentage of detecting pre-cancerous cells was calculated in both methods. The results were then analysed using paired t test considering significance at level of $p<0.05$.

Ethical approval

Ethical approval for this study was obtained from the Ethics Review Committee of the Faculty of Allied Health Sciences, University of Ruhuna, Galle, Sri Lanka (Clearance No: 221.05.2023).

Table 2: Defined scoring system for cytomorphological parameters

Cytomorphological parameter	**Scores**		
	01	**02**	**03**
Cell/cytoplasmic boarders	Indistinct	Somewhat distinct	Distinct
Cytoplasmic staining	Unsatisfactory	Satisfactory	Excellent
Nuclear boarders	Indistinct	Somewhat distinct	Distinct
Chromatin staining	Hazy	Somewhat distinct	Distinct with crisp chromatin

Results

A total number of 152 paired smears were included in the study. Interestingly all the smears recognized as ASCUS and LSIL by the standard method, were recognized by the modified method as well. The detection rate of ASCUS and LSIL were 5.9% and 1.3% respectively (Table 3).

98.7% (150/152) cervical smears stained using the conventional method showed distinct cytoplasmic borders, while two smears (1.3%) showed somewhat distinct borders. 96.7 % (147/152) smears stained with the modified method showed distinct cytoplasmic borders, and five smears (3.3%) had somewhat distinct borders. Notably, none of the smears stained using either method had indistinct cytoplasmic borders.

On assessment of the quality of cytoplasmic staining, 60 smears (39.5%) stained using the conventional method were rated as excellent and 92 smears (60.5%) were rated as satisfactory. For the modified method, 65 smears (42.8%) were rated as excellent and 87 (57.2%) as satisfactory. Importantly, all smears from both methods showed clear nuclear details (Table 4). No significant difference was observed between the cytomorphological parameters assessed using both methods ($p > 0.05$) (Table 5).

Quality index of the modified method was 0.911, whereas it was 0.932 for the conventional method (Table 6).

Images of smears stained using both methods are shown in figures 1 and 2 .

Table 3: Comparison of detecting pre-cancerous cells by standard and modified methods

Method	**Standard**		**Modified**	
	No:	**%**	**No:**	**%**
ASCUS	9/152	5.9%	9/152	5.9%
LSIL	2/152	1.3%	2/152	1.3%
HSIL	Not detected			
Atypical glandular cells	Not detected			

Table 4: Comparison of the frequencies of scores in standard and modified method

Score	**01**		**02**		**03**	
	No:	**%**	**No:**	**%**	**No:**	**%**
CBM	-	-	5	3.3	147	96.7
CBS	-	-	2	1.3	150	98.7
CSM	-	-	87	57.2	65	42.8
CSS	-	-	92	60.5	60	39.5
NBM	-	-	-	-	152	100
NBS	-	-	-		152	100
NSM	-	-	-	-	152	100
NSS	-	-	-	-	152	100

(CBM- cytoplasmic borders stained by modified method, CBS- cytoplasmic borders stained by standard method, CSM- cytoplasmic stain stained with modified method, CSS- cytoplasmic stain stained by standard method, NBM- nuclear borders stained by modified method, NBS- nuclear borders stained by standard method, NSM- nuclear stain stained by modified method, NSS- nuclear stain stained by standard method)

Table 5: Comparison of the frequencies of scores in standard and modified method

Cytopathological parameter assessed	t value	Sig (2 tailed)
Cytoplasmic borders(modified)- cytoplasmic borders(standard)	-1.765	0.800
Cytoplasmic stain(modified)- cytoplasmic stain(standard)	-0.390	0.697
Nuclear borders(modified)- nuclear borders(standard)	0.006	0.995
Nuclear stain (modified)- nuclear stain(standard)	-1.827	0.770

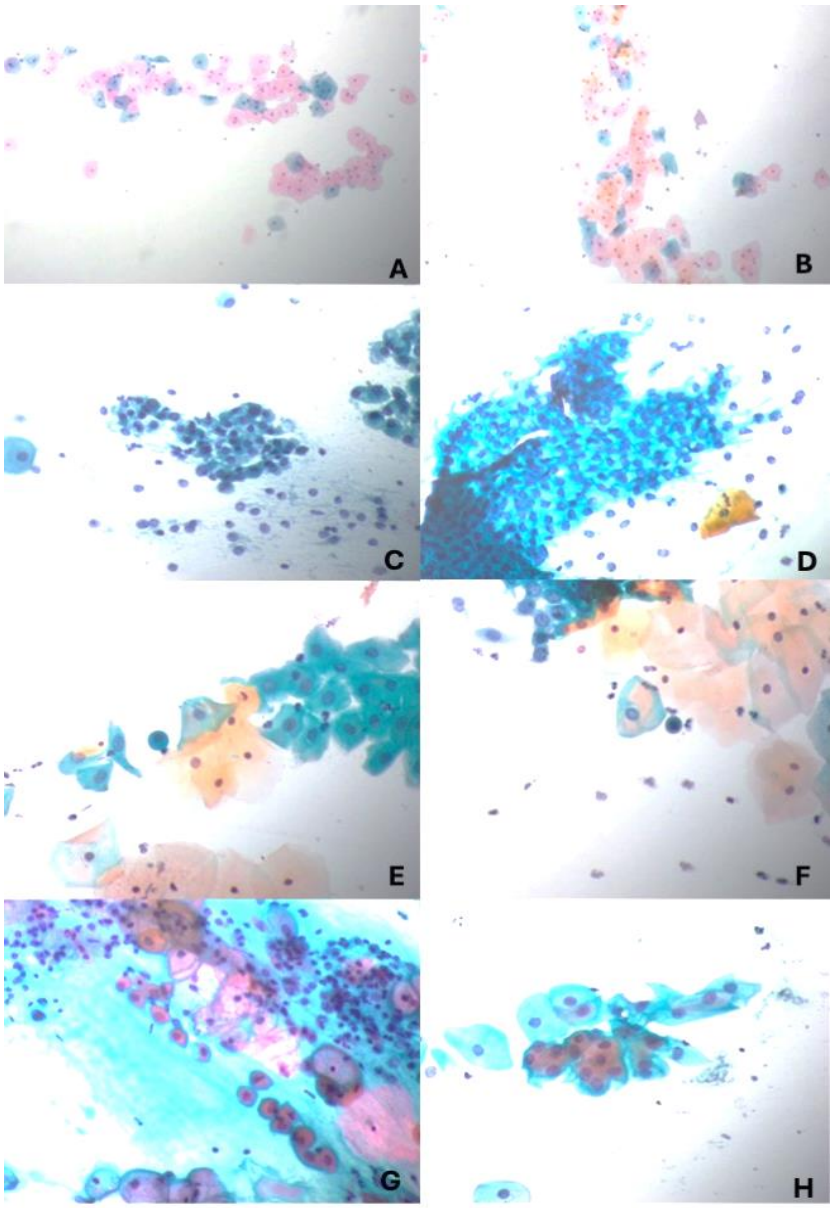

Figure 1 A) Superficial and intermediate cells stained by standard method (×100) B) Superficial and intermediate cells stained by modified method (×100) C) Endocervical cells stained by standard method (× 100) D) Endocervical cells stained by modified method (×100) E) Parabasal cell stained by standard method (×400) F) Parabasal cell stained by modified method (×400) G) LSIL stained by standard method (×400) H) LSIL stained by modified method (×400)

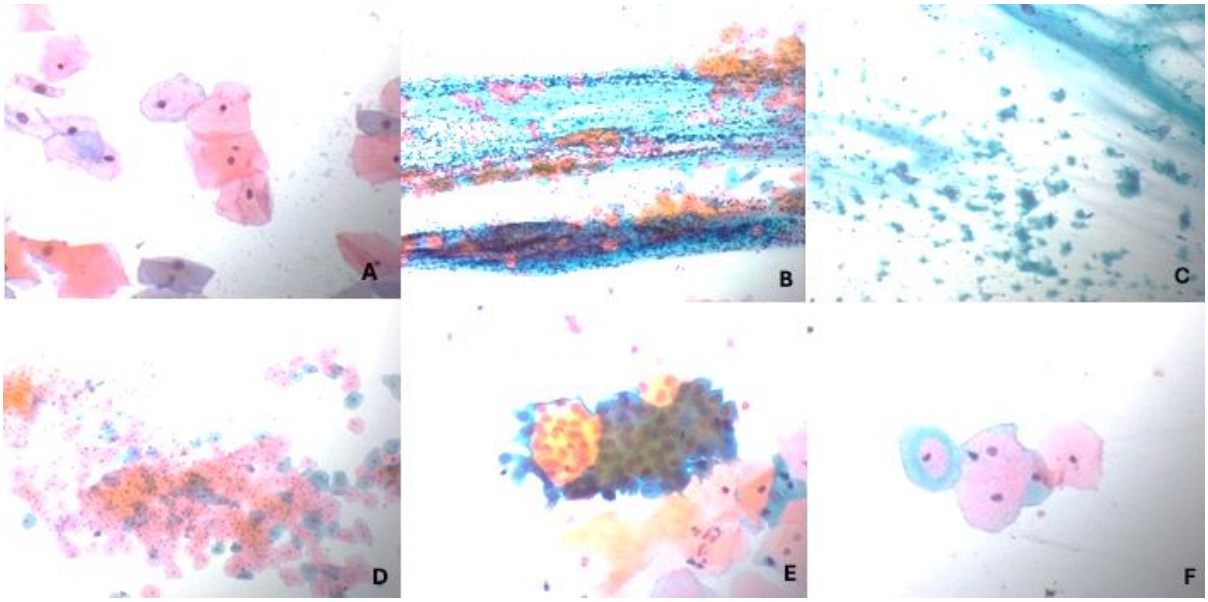

Figure 2 Examples of smears stained by modified method A) Well-defined nuclear and cytoplasmic staining pattern with clear cytoplasmic boarders (×400) B) Heavy inflammation (×100) C) Cytolysis (×100) D) Cell overlapping stained (×100) E) Clustered cell ball of endocervical cells with poor staining (×400) F) Superficial cell stained as green (×400). Poorly stained cells were seen in both methods.

Table 06: Comparison of Quality Index of the Standard and the Modified method

Method	**Modified Papanicolaou method**	**Standard Papanicolaou method**
Total cases	152	152
Possible maximum score	12*152= 1824	12*152=1824
Obtained score	10.93*152= 1662	11.18*152= 1700
QI	1662/ 1824= 0.911	1700/1824= 0.932
Maximum QI	1.000	1.000

Table 07: Alcohol Usage and Cost: Standard vs. Modified Staining Methods

Standard method	**Modified method**
Amount of Alcohol 6 litres	Amount of Alcohol 1 litre
Cost of 1 litre of Alcohol 3750.00 LKR	Cost of 1 litre of Alcohol 3750.00 LKR
Total cost for Alcohol 3750.00*6= 22500.00 LKR	Total cost for Alcohol 3750.00*1= 3750.00 LKR

Cumulative cost per slide

Six litres of absolute alcohol are required for the standard method to prepare alcohol solutions for hydration, dehydration and rinsing. The modified method requires one litre. Therefore, there is a saving of 18,750 LKR from only one run of the modified method. The cost was reduced by 80% due to the limited alcohol use. For the modified Papanicolaou method, the total protocol duration was approximately 10 minutes, whereas the standard method required approximately 20 minutes to complete all staining steps.

Discussion

Of the 152 paired smears included in the study, 92.8% were negative smears of intraepithelial lesion or malignancy, 5.9% showed ASCUS, and 1.3% showed LSIL, according to both conventional and modified methods. Interestingly, all the smears recognized as ASCUS and LSIL by the conventional method were recognized by the modified method as well. According to a study done by Gachie et al in 2011, the detection rates of ASCUS, LSIL, and HSIL were 1.25%, 1.89%, and 1.25%, respectively(4). In another similar study conducted by Gupta et al. in 2009, the detection rates were 7%, 3%, and 1% for ASCUS, LSIL, and HSIL, respectively, in the modified method(22).

In the present study, all the smears stained with the modified and conventional methods had distinct nuclear borderers and distinct-crispy chromatin staining. Most of the conventional pap smears (98.7%) showed distinct cytoplasmic borders with only two (1.3%) showing somewhat distinct cytoplasmic borders. In the modified method, 96.7% showed distinct cytoplasmic borders and five (3.3%) showed somewhat distinct cytoplasmic borders. Neither method resulted in indistinct cytoplasmic borders. In the conventional method, 60.5% of smears exhibited satisfactory cytoplasmic staining, while 39.5% showed excellent staining. In comparison, the modified method resulted in 57.2% satisfactory and 42.8% excellent cytoplasmic staining. A previous study by Biswas et al. in 2008, in which alcohol was replaced by 1% acetic acid, reported that 90% cases showed optimal cytoplasmic staining and 95% showed optimal nuclear features. A similar study done by Gupta et al. in 2010 reported 79.6% cases with distinct and 20.4% with indistinct cytoplasmic borderers and 21.3%, 58.3%, and 20.4% cases with excellent, satisfactory, and unsatisfactory cytoplasmic staining, respectively. 75.6% cases showed distinct and 24.2% showed indistinct nuclear borders, while 73.3% cases exhibited chromatin with crisp nuclear pattern and 26.7% showed hazy nuclear staining(16,22).

The staining quality of the stained smears were compared using four cytomorphological parameters. There was no statistically significant difference between cytoplasmic boarders, cytoplasmic staining, nuclear boarders, and nuclear staining using the standard method and modified method ($p<0.05$). A study done by Gachie, et al in 2011, which replaced alcohol with 1% acetic acid, found that there was no significant difference in the staining of cytoplasmic and nuclear borders. However, this study reported a significant difference in the staining of the cytoplasmic and nuclear chromatin compared to the standard method(5). Another study by Salazar and Zumaran in 2018 reported no significant difference between the eco-pap and the standard method(23).

Staining quality was compared by using the quality index, and it showed that there was no considerable difference between the QI of standard and modified Papanicolaou staining methods (0.932 in the standard method and 0.911 in the modified method). Salazar and Zumaran reported a QI of 0.94 using a modified method (23), whereas a study by Moosa et al. which replaced alcohol with acetic acid, reported a lower QI of 0.82, (21).

The modified method used in this study was more cost-effective than the standard method. Six litres of absolute alcohol are required in the standard method to prepare hydrating, dehydrating, and rinsing alcohol solutions. However, only 1 litre of absolute alcohol is needed for the modified method. Therefore, a considerable amount of money can be saved in a single run of the modified method. Interestingly, 0.5% aqueous HCL and Scott's

tap water are also not used in the modified method, which further reduces the cost.

Conclusion

In conclusion, this modified Papanicolaou staining method which requires less amount of alcohol, no aqueous HCL and no Scott's tap water is a simple and user-friendly method which does not compromise the staining quality, preservation and diagnostic standards. It can be used as an alternative to the standard Papanicolaou staining method in resource limited settings.

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