RESEARCH ARTICLE

Comparison of Cervical Cell Morphology Using Two Different Cytology Techniques for Early Detection of Pre-Cancerous Lesions

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Abstract

Cervical cancer is an issue of foremost importance globally, specifically affecting the developing nations. Significant advances have taken place with regard to diagnosis of cervical cancer, especially with screening. Appropriate screening measures can thus reduce the incidence of cervical cancer. The most desirable screening technique should be less invasive, easy to perform, cost-effective and cover a wide range of diagnostic icons. Manual liquid based cytology (MLBC) can be considered as one of the suitable technique for screening with the above-mentioned benefits. The aim of the current study was to compare two cervical screening techniques on the basis of different morphological parameters and staining parameters by using modified acetic acid Pap staining to see the possibility of reducing time economy involved in conventional Pap staining (CPS). The study was conducted on a total 88 cases and all were analyzed with both MLBC and CPS. Forty eight cases that were regarded as satisfactory on the basis of Bethesda system by both methods were further recruited for investigation. Their morphological parameters and staining quality were compared and scored according to a scoring system defined in the study. Quality indices was calculated for both staining procedures and smear techniques.

Keywords: Cervical cancer - screening - staining - cytology - manual liquid based cytology - Pap smear

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Introduction

Cervical cancer is one of the foremost public health concerns after the breast cancer. Eighty-three percent of the global cervical cancer occurs in developing countries where its mortality rate is approximately 80% of 470,000 patients (Parkin et al., 2002). Its involvement in overall cancer burden is substantial in all societies whether developed or developing nations. Cervical cancer causes 270,000 deaths globally per year, major portion of these mortalities belongs to underdeveloped countries (Ferlay et al., 2010).

Cervical cancer screening has reduced cervical cancer and its mortality rate up to certain extent (Hicks et al., 2006). The major contributing factors responsible for cervical cancers are sexual activities, lack of education and infectious etiology including Herpes simplex virus, Human Papilloma virus, and HIV (Chih-Ming et al., 2004). Cervical cancer can be prevented by primary as well as secondary measures. Secondary prevention through cytological examination has been the mainstay for early detection of cervical cancer (Nandini et al., 2012). Cervical changes can be perceived much before its progress into invasive carcinoma and this is the basis of cytological screening (Kavatkar et al., 2008). Cytological examination could be very helpful for timely diagnosis of cervical cancer, but this preventive measure is not commonly practiced in developing countries due to scarcity of resources, technical personals and other facilities (Nandini et al., 2012). The reason behind low screening settings in developing countries is little or absolutely no conception of precautionary measures, economic hindrance, social and cultural issues and of course lack of knowledge (Blanks et al., 2007). In Pakistan, the burden of disease is increased, cervical cancer is the 4th common cancer in Pakistani women with an age standardized incidence rate (ASIR) of 6.5 per 100,000 (Sardar et al., 2008).

Number of studies revealed that despite of the higher socio-economic status of South Asian females , the Pap screening is very uncommon. Therefore, the major reason is not low economic resource but lack of awareness (Gupta et al., 2002; Chaudhry et al., 2003; Sutton et al., 2004). Awareness of the population about cervical cancer screening could lead to encouraging steps towards

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implementation of screening setup in Pakistan (Sardar et al., 2008).

The Pap smear test can diagnose cervical cancer during precancerous conditions therefore it is mostly used as screening tool in developed nations where this test reduce incidences as well as mortality associated with cervical cancer (Nygard et al., 2002; Hewitt et al., 2004; Canfell et al., 2006). Cervical screening by using conventional Pap method rivets the microscopic examination of cell samples taken from the ecto- and endocervix, directly on glass slides and subsequent staining by using the Pap procedure (King et al., 1992).

Timely cervical screening by using Pap method has reduced approximately 50-70% death rate associated with invasive cervical carcinoma, but its sensitivity is affected significantly in the presence of blood cells, mucous and areas of overlapping epithelial cells during specimen collection (Sherwani et al., 2007; Kavatkar et al., 2008). Most parts of slide when prepared for regular Pap smear contain cell debris, inflammatory cells and sheets of epithelial cells that can interfere with correct diagnosis.

Secondly, delay in fixation of smear after taking sample may leads to deformation of cellular morphology. Cells in fact relocating on slide do not correspond to the total number of cells collected, many of them left on the device Xian (2011). Interpretation of results needs technical expert that are hardly available in developing countries. The problems associated with the conventional Pap smear lead to the developments of advanced technologies such as Thin Prep and Sure Path most commonly used in developed countries like UK and USA (Kavatkar et al., 2008; Deshou et al., 2009). Residual sample after LBC can be further utilized in the detection of HPV (Sherwani et al., 2007; Kavatkar et al., 2008). However the techniques, tools, and supplies used for liquid-based methods are costly for resource limited settings (Judy et al., 2006).

Alternately, manual liquid based cytology for cervical screening could be the most appropriate choice (Johnson et al., 2000). The universal stain for cervical cytological screening is Papanicolaou stain. It gives a polychromatic, transparent staining reaction with crisp nuclear and cytological features (Bibbo, 1991). However it utilizes a considerable amount of ethyl alcohol and it is time consuming (Sato et al., 2004). Ethyl alcohol can be replaced by acetic acid with certain modification in conventional pap staining procedure that could be economical and may give satisfactory results in lesser time (Biswas et al., 2008).

Materials and Methods

The current study was conducted on 80 patients from Get Well medical clinic, G-10 Islamabad, Pakistan. Samples were collected with the help of vaginal speculum and cervical broom with detachable head that takes samples from endo as well as ectocervix at a time. The extended central bristles were inserted into cervical canal and short bristles on sides were positioned to take samples from ectocervix. Broom was completely rotated at 360° for five times. Cells collected were transferred on two differently labeled microscopic slide by paint-stroke like motion with one side of brush first and then from other side of brush on same slide. Second slide was also prepared in same manner from same patient. Slides were then fixed immediately with fixative. As study was based on split-sample method, the head of the broom was detached and placed in the pre sterilized and labeled bottle containing preservative prepared in lab of IBMS, KMU, Peshawar. The preservative contained Sodium chloride, sodium citrate, 10% formalin and alcohol (Kavatkar et al., 2008).

Specimen were collected in three different places i.e. two on microscopic slides and one in preservative solution form single patient. Samples were then brought to lab in IBMS for further processing. Specimen were subjected to three different methods named manual liquid based cytology, conventional Pap staining and acetic acid Pap staining.

Manual liquid based cytology

Two slides were prepared from samples carried in preservative from clinic by following manual liquid based cytology procedure. One was stained with standard Papanicolaou stain and other was stained with acetic acid Pap staining. MLBC slides were prepared by the procedure stated in two different studies (Nandini et al., 2012; Kavatkar et al., 2008).

After MLBC we had total four slides from each patient. Two slides obtained directly from clinic and two slides with polymeric film prepared after manual liquid based cytology. One slide from scrape smear and one slide of polymeric film were subjected to standard Papanicolaou staining, while second scrape smear slide from clinic and other polymeric slide after MLBC were subjected to modified acetic acid Pap staining (Biswas et al., 2008).

The samples were blind examined by the supervisor and one other histo-pathologist and interpreted individually. For evaluation of both techniques i.e. conventional Pap smear and manual liquid based cytology, the

Table 1. Morphological Parameter with Defined Scoring for Comparison

Morphological Parameters	Scoring			
	0	1	2	3
Cellularity	No cells	Scant cells	Adequate cells	Abundant cells
Clear Background	Abundant debris present	Debris present but adequate for diagnosis	Clear background	---
Uniformity of distribution	Cell restricted to only one area of slide	Cellular clumps through slide adequate for diagnosis	Few areas of cells	Cells uniformly distributed on slide
Artifacts	Absent	Interfering but acceptable	Clear	---
Cellular overlapping	Cells only in clumps	Few regions of clumps	Minimal overlapping	---
Folded cytoplasmic borders	Many ≥ 25%	Few ≤ 25%	Absent	---

Table 2. Nuclear Parameter with Defined Scoring for Comparison

Staining Parameters	Scoring		
	0	1	2
Nuclear Staining	Indistinct+Hazy	Distinct+Hazy OR Indistinct+Crisp	Distinct+Crispy
Cytoplasmic staining	Poor	Optimal	Transparent

Table 3. Comparison of MLBC with CPS for Diagnostic parameter

S.NO	Selection parameters		No of cases	
			CPS	MLBC
1	Intact Membrane			66
2	Satisfactory (Bethesda 2003)		47	50
3	Unsatisfactory (Bethesda)		19	16
4	Smear	Normal	25	29
		Inflammtory	22	18

morphological parameters were scored from 0 to 3 shown in Table 1 on the basis of criteria defined in study conducted by (Dwivedi et al., 2012).

Staining quality of four different techniques named standard Pap staining, MLBC Pap staining, acetic acid Papanicolaou and MLBC acetic acid pap staining were assessed by nuclear and cytoplasmic staining quality with scoring from 0 to 3 given in Table 2. (Alves et al., 2004; Biswas et al., 2008).

Quality index

After individual evaluation of screening techniques on the basis of morphological and diagnostic parameter and subsequent assessment of staining techniques through nuclear and cytoplasmic staining, overall evaluation of best techniques among smear preparation and staining was done by means of quality index originally devised by Chan et al., 1988. The maximum score possibly attained by one case, after keeping in view for all of the six morphological parameters could be 14 and two staining parameters , could be 4. One the basis of single case the highest attainable score with one technique was calculated by multiplying the number of subjects in study with maximum possible score for each evaluation. A "Quality Index" was then found by the ratio of obtained score by maximum score: *Quality index=actual score obtained/maximum score possible*.

Results

Diagnostic parameters

Total 80 cases were selected for screening study and while performing MLBC, membrane remained intact in 66 cases and was disrupted in rest of 14 cases. Sixty six cases with intact membrane were categorized as satisfactory and unsatisfactory on the basis of Bethesda system (Solomon et al., 2001). While doing MLBC fifty cases were satisfactory and out of remaining, fourteen contained more than 75% inflammatory infiltrate and blood however, two cases are lack of transformation zone. Duplicate slides of same 66 patients from gynecology clinic were subjected to CPS cytology, out of which forty-seven cases were satisfactory and remaining nineteen were unsatisfactory.

In the whole forty-seven cases that were regarded as satisfactory on the basis of both MLBC and CPS were sorted only for normal smear and inflammatory smear for comparison of two techniques. Eighteen cases were reported with inflammatory smear and twenty nine cases with normal smear when performed with MLBC, whereas twenty two cases were seen with inflammatory smear and 25 were diagnosed as normal smear with CPS shown in Table 3.

Morphological parameters

Cellularity

Adequacy: in current study normal conventional Pap Smear (CPS) contains fewer well preserved superficial squamous cells and intermediate squamous cells with normal morphology however; in Manual Liquid Based Cytology (MLBC) stained by both acetic acid as well as

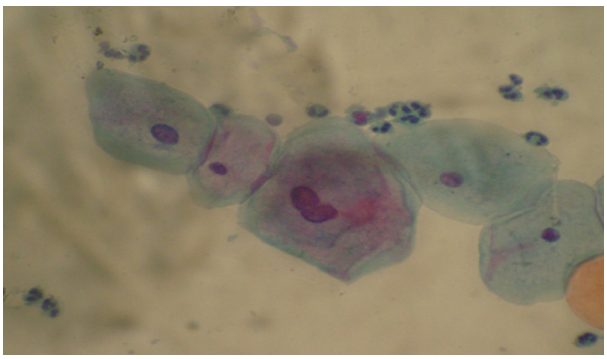

Figure 1. Micrograph with 40X Objective and 22 Eyepiece Showing Cellular Count ≥ 5000 for Normal Smear Cellularity in MLBC Slides.

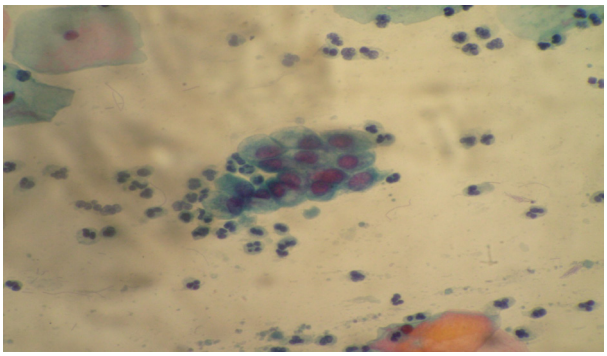

Figure 2. Inflammatory Smear Cellularity in MLBC Slides

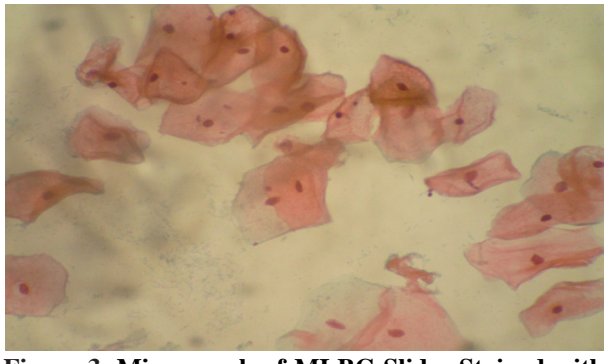

Figure 3. Micrograph of MLBC Slides Stained with Modified Acetic Acid Pap Staining to Show Uniformity of Distribution, Clarity of Background

Table 4. Quality Index of Screening and Staining Techniques

Scoring Index	Screening method		Staining Method			
	CPS	MLBC	Modified acetic acid Pap Staining	Conventional Pap Staining	MLBC Pap Staining	MLBC Acetic cid Pap staining
Obtained Score	371	429	217	156	176	205
Maximum Score	658	658	264	264	264	264
Quality Index	0.563	0.651	0.82	0.59	0.66	0.77

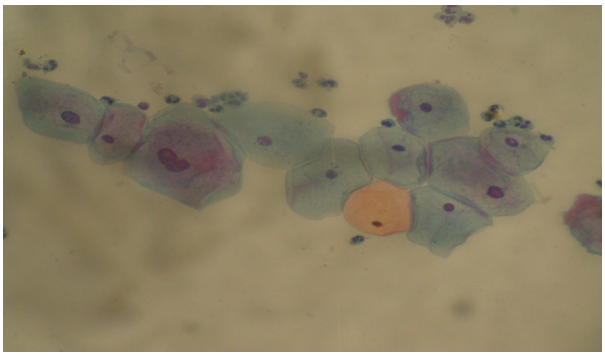

Figure 4. Micrograph of MLBC Slides Stained with Regular Papanicolaou Staining with Score 2 for Cellular Overlapping

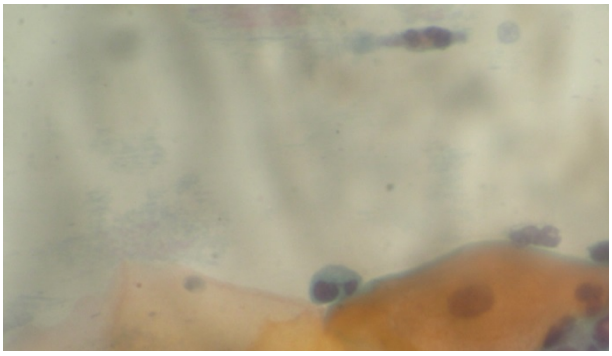

Figure 5. Micrograph of MLBC Slides Stained with Modified Acetic Acid Pap Staining with Score 1 for Cellular Overlapping. Age-specific

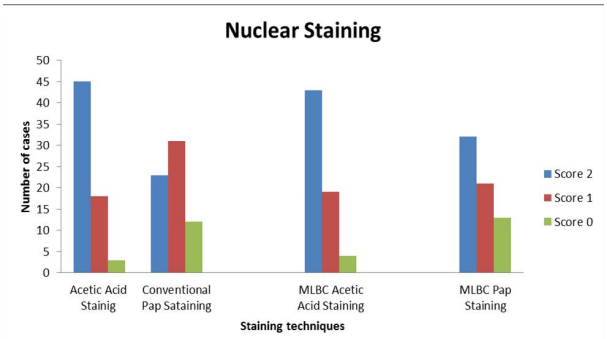

Figure 6. Scoring for Quality of Nuclear Staining by Using Different Techniques.

Pap staining, there is increased cellularity for both types of well-preserved cell that improves the chances of adequacy for smear selection in Bethesda scrutiny revealed by Figure 1. However in case of Inflammatory smear CPS is not free from obscuring blood and inflammatory cells whereas MLBC gives more clear picture. When slides of MLBC smear follows acetic acid staining, they give results with even more clear background as shown in Figure 2.

Cellular count: recommended cellular count for sample selection is ≥5000 for MLBC according to American School of Pathology and ≥8000 for CPS. All samples smears that were selected contained enough cell count to satisfy Bethesda system. Most of the samples contains the cells from 4 to 6 cells in field with 40× objective and 22 eye piece, which means they contains more than 5000 cells Figure 1.

Uniformity, background and artifacts

Table 5. Individual Scores of Morphological Parameters and Quality Index of Techniques

Morphological Parameters	Conventional Pap Smear	Manual liquid based cytology
Cellularity		
No cells	0	1
Scant cells	2	2
Adequate cells	43	33
Abundant cells	2	11
Total	94	101
Clear Background		
Abundant debris present	0	0
Debris present but adequate for diagnosis	28	7
Clear background	19	40
Total	66	87
Uniformity of distribution		
Cell restricted to only one area of slide	0	3
Cellular clumps through slide adequate for diagnosis	9	5
Few areas of cells	15	8
Cells uniformly distributed on slide	23	31
Total	108	114
Folded Cytoplasmic Borders		
Inadequate for diagnosis	11	6
Interfering but acceptable	30	33
Absent	6	8
Total	42	49
Cellular overlapping		
Cells only in clumps	2	1
Few regions of clumps	29	14
Minimal overlapping	16	32
Total	61	78
Obtained Score	371	429
Maximum Score	658	658
Quality Index	0.563	0.651

Table 6. Individual Scores of Staining Parameters and Quality Index of Four Staining Techniques

Staining parameters	Acetic Acid Staining	CPS	MLBC Acetic Acid Staining	MLBC Pap Staining
Nuclear staining				
Indistinct+Hazy	3	12	3	13
Distinct+Hazy/Indistinct+Crisp	18	31	18	21
Distinct+Crispy	45	23	45	32
Total	108	77	108	85
Cytoplasmic Staining				
Poor	6	11	5	9
Optimal	11	31	25	23
Transparent	49	24	36	34
Total	109	79	97	91
Obtained Score	217	156	205	176
Maximum Score	264	264	264	264
Quality Index	0.82	0.59	0.77	0.66

Table 7. Average Score and p Value of Different Morphological Parameters

Methods	Distribution		Cellular Overlapping		Folded Cytoplasmic Borders		Clear Background	
	CPS	MLBC	CPS	MLBC	CPS	MLBC	CPS	MLBC
Av: Scoring	2.28	2.43	1.29	1.68	0.87	1.06	1.36	1.87
p value	0.47		<0.001	0.108			<0.001	
S.E.D.	0.1765		0.1063	0.1179			0.0869	

When compare three systems MLBC stained with acetic acid give best results in view of three parameters uniformity of distribution, clear background and artifacts as shown in Figure 3, however, MLBC followed by Pap staining give intermediate results as revealed by Figure 4.

Cellular overlapping and folded cytoplasmic boarders

MLBC shows little to moderate cellular overlapping having score 2 shown in Figure 4. When compared with CPS that gives cellular overlapping with score 1 in more than 80% of cases, Figure 5 However, there is no significant difference in cytoplasmic border folding in both methods. Quality index for both screening technique is given in Table 4. However, individual scores of each morphological parameter is given in Tables 5 and 6.

Staining parameters

Staining of four methodologies was compared on the basis of scoring for two parameters i.e. nuclear and cytoplasmic staining. Acetic acid papanicolaou staining technique gives maximum scores i.e. 45 good category, 18 satisfactory and 3 unsatisfactory defined in material and methods for nuclear staining as shown in Figure 6. however, scores were 47 excellent, 14 optimum, and 5 poor for cytoplasmic staining given in Figure 7. Remaining three staining techniques are categorized as modified acetic staining of MLBC slides, MLBC stained by Pap stain and CPS in descending order based on the scoring index of staining quality shown in Table 4.

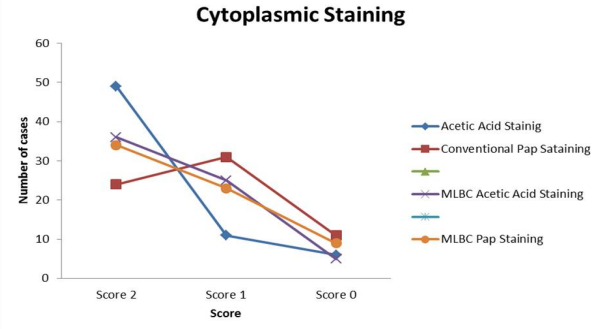

Figure 7. Comparison between Four Staining on the Basis of Scoring for Cytoplasmic Staining

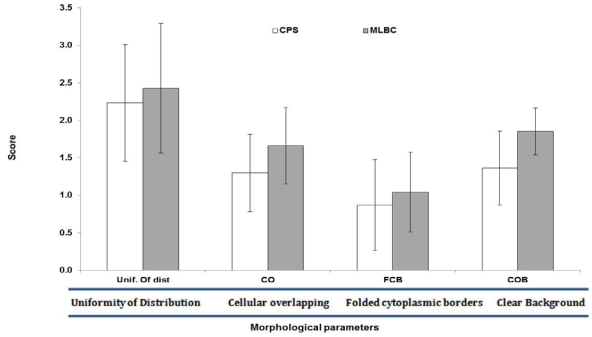

Figure 8. Av: Score of Different Morphological Parameters by Using Different Methods

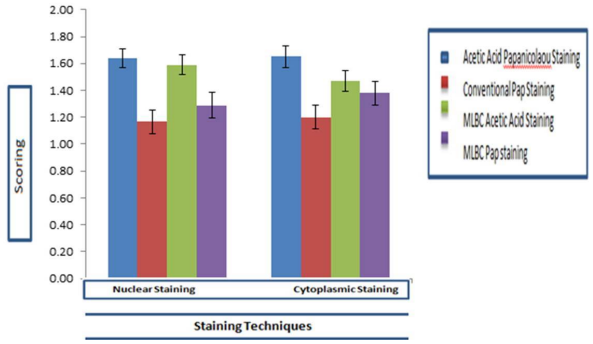

Figure 9. Av: Score of Nuclear and Cytoplasmic Parameters by Using four Different Staining Methods

Statistical analysis

Morphological parameters like uniformity of distribution and folded cytoplasmic borders have P value greater than 0.005 (p>0.005) showing no significant difference between two techniques. i.e. CPS and MLBC with P value 0.471 and 0.108 respectively by using ANOVA. However, p<0.005 for both cellular overlapping and cleanliness of background showing highly significant difference between two techniques as shown in Table 7. Average scores of all four parameters are shown in Figure 8.

Statistics involved in comparison of both nuclear and cytoplasmic staining with all four staining techniques is highly significant with p<0.005 for cytoplasmic staining and p<0.005 for nuclear staining by using ANOVA. The average score is shown in Figure 9.

Discussion

Number of studies were performed for comparison of Conventional Pap smear and Manual liquid based cytology by using standard Pap staining in subsequent steps of staining. However, in current study both techniques were compared by using standard Pap staining method and modified acetic acid Pap staining individually on the basis of different morphological parameters, nuclear staining and cytoplasmic staining. Despite of extensive use of CPS for cervical screening this method has certain shortcomings (Deshou et al., 2009). Maksem et al., suggested the new method in 2001 that could possibly reduce these problems, this new method involves the use of polymeric membrane and named as Manual liquid based cytology. (Maksem et al., 2001). In this method membrane is formed from polymeric solution containing Carbowax and, agrose (Maksem et al., 2006) in which cells are suspended. Polymeric film is then applied on microscopic glass slide (Maksem et al., 2001).

In current study we have compared the clarity and overall beneficial aspects of MLBC technique and

conventional Pap smear test in terms of scoring that could lead us to a better method for diagnosis. In present study different factors like cost effectiveness, time economy and better preservation of marked nuclear as well as cytoplasmic features of modified acetic acid pap staining are amalgamated with advantageous features of MLBC techniques which was then compared with simple Pap smear test, acetic acid papanicolaou staining and conventional Pap staining to map out for the most acceptable technique which could be better for cervical screening with respect to time economy, cost, clarity of features and of course correct diagnosis.

In our study 27% cases were unsatisfactory according to CPS and 24% cases were found unsatisfactory with MLBC due to scant cell, presence of blood and mucous. The percentage of unsatisfactory smears is much lower in studies like in Bergeron et al., reported (0.14%), Garbar et al., 2005 found (0.9%). The only possible reason behind high percentage in our study could be the use of expensive automated methods in those studies, however in current study manual method was used for membrane preparation.

In LBC, the sample is first placed in fixative and then proceed for further processing instead of making slides directly as in CPS, thereby cellular structure are more well preserved and reduced drying artifacts as cells are immediately fixed Brud (2003). According to one study LBC enhance the specimen adequacy, decrease the count of unsatisfactory smears and improves screening results (Celik et al., 2008). Liquid based cytology also improves adequacy by increase in cellularity (Nandini et al., 2012). Similarly, in current study cellular structure are more well preserved in MLBC slides as compare to CPS, therefore MLBC gave more clear picture of cellularity which is beneficial for correct diagnosis. In MLBC there is marked decrease in artifacts, contaminating mucus and blood. Cells are evenly distributed on slides and centrifugation in this method offers a proper mixing (Afaf et al., 2012). Likewise in present study the average scoring of morphological parameters like cellular overlapping, clarity of background and artifacts is significantly different in both techniques. Manual liquid based cytology gives more clear results with clear background, less artifacts and lesser degree of cellular overlapping when compared with conventional Pap smear. However folded cytoplasmic borders are observed equally in slides prepared by both techniques and no significant difference is observed between these two techniques for folded cytoplasmic borders statistically. Cells are evenly distributed in slides prepared by MLBC as compared to Pap smear test in current study.

Visual inspection with acetic acid (VIA) has been extensively used for cervical cytology in economically poor countries, which encourages the use of acetic acid in staining of Pap smear slides in this study. In modified acetic acid papanicolaou, staining 1% acetic acid is used instead of ethyl alcohol in various steps. Acetic acid is used as dehydrating agent and it is economical and easily available (Dighe et al., 2006). In this study, all four techniques named acetic acid papanicolaou staining, conventional Pap staining, MLBC acetic acid staining and MLBC Pap staining are compared on the basis of two staining parameters i.e. nuclear staining and cytoplasmic staining. All four staining methods gave maximum number of excellent scoring followed by optimal and then poor score in cytoplasmic staining. But the result of average score of cytoplasmic staining was significantly different statistically due to number of cases in excellent, optimum and poor category. Excellent staining revealed maximum transparent cytoplasm, poor might be due to lesser penetration in thick part of cytoplasm (Biswas et al., 2008)

In techniques which use membrane method, overall the result was clear as compare to acetic acid papanicolaou staining and CPS because membrane method removed unnecessary waste material like blood, mucous and debris and as cellular overlapping is also very few in this method, stain was evenly distributed in cytoplasm and features are more prominent. As for as nuclear staining is concerned acetic acid papanicolaou staining gave sharp nuclear features and crisp nuclear chromatin in maximum number of cases, however there were moderate number of satisfactory cases which are acceptable for diagnosis and only few cases were unsatisfactory but overall average score is maximum for modified acetic acid papanicolaou staining. In satisfactory score, chromatin is mildly hazy with little diffused nuclear features. MLBC acetic acid gave highest results in good category as in acetic acid papanicolaou staining but few in number followed by satisfactory and unsatisfactory category in descending order, rest of two staining methods MLBC Pap and CPS gave overall acceptable score for correct diagnosis.

By using two different methods for screening, some morphological features attained maximum score by using one techniques, while others got equal score by both techniques. To decide the overall favorable screening technique, quality index was calculated that was based on the ration of individual score to the maximum attainable score by considering all five morphological parameters. The quality index of MLBC is higher with value of 0.65 as compare to CPS having value 0.56, which means MLBC is more superior techniques for screening as compare to CPS. However, for quality index for staining quality of cytoplasm and nucleus, modified acetic acid staining method is very favorable with index of 0.82 followed by MLBC with modified acetic acid pap staining having index value 0.77. Conventional Pap staining is acceptable with index score of 0.59.

In conclusion, it is concluded from present study that MLBC methodology could be used for cervical screening in low resources setting instead of liquid based cytology and the slides prepared by MLBC can be stained with modified acetic acid pap staining, this can compensate the time and the cost additionally required by MLBC instead of conventional pap smear test.

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