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# Comparison of Conventional PAP smear and Manual Liquid Based Cytology with P16 marker in screening for cervical cancer in Indian tertiary healthcare setups in Mysuru

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**TITLE**

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**INTRODUCTION**

Cervical cancer ranks the fourth most common cancer affecting females worldwide, after breast, colorectal and lung cancer. There are about 5, 28,000 new cases of cervical carcinoma reported every year.[1] . Around 85% of the new cases occurred in less-developed countries.[2] In resource-rich countries, there was a decrease in the incidence of cervical cancer after the introduction of Pap testing. Of late, this decrease has become less evident, and frequent retesting is required to attain an acceptable sensitivity because of the low sensitivity of the Pap test. [3]

In India, there are 20.2 per 100000 new cases of Cervical Cancer diagnosed and 11.1 per 100000 deaths annually accounting for more than one fifths of global cervical cancer deaths. [4] The standardized age wise rate of incidence of this cancer in Indian population based registries varied from 13 to 25 out of every 100,000 women [5]

Owing to a lack of a national immunization program including human papillomavirus (HPV) vaccination and decreased accessibility of cervical cancer screening, the disease is characterized by late detection, lack of access to affordable and quality health care, and high mortality rates. [6]

In Sub-Saharan Africa, 34.8 of every 100000 women are diagnosed with cervical carcinoma annually and 22.5 out of 100000 women succumb to this disease. Therefore, various economical methods are needed apart from conventional pap smear screening to detect cervical cancer in low resource settings. Alternative methods are being introduced in low and middle income countries (LMIC) to enhance early detection, at low cost, manual liquid based cytology (MLBC) being one of them.[4]

Papanicolaou Smear Test was a novel invention by Dr George Papanicolaou in the year 1927 which gained maximum popularity in 1950 as a screening test for Cervical Cancer. Liquid based cytological technique has been recently developed and has gained popularity because in preliminary studies the use of such techniques was associated with a reduction in incidence of inadequate cervical smears. [7,8]

The conventional Papanicolaou smear (CPS) has been the central component of screening for cervical cancer and its precursor lesions for about half a century without major changes in the techniques with regards to preparation and interpretation. In spite of its success as a preventive screening tool for cervical cancer, CPS has its limitations [9]. False negatives in CPS may be related to insufficient sampling, ineffective transfer of the sample onto the glass slide or

errors in the microscopic assessment of the slide [8]. Although a clinician may have impeccable collection and sampling technique, only about 20% of the cells collected get smeared on the glass slide in CPS [10, 11,12]

In order to solve these problems, a new technique of slide preparation namely the Manual Liquid Based Cytology (MLBC) was introduced by [10]. Many workers have come up with indigenous methods of manual liquid based cytology, to increase its cost efficiency. In the manual liquid based method, cells are uniformly dispersed by a membrane, from a suspension of cells in a polymer solution [10, 13]. It is a technique that enables cells to be suspended in a monolayer and thus improves detection of precursor lesions and of specimen adequacy. Thus it improve the effectiveness of cervical cancer screening in a population by increasing the detection of histologically confirmed neoplastic and pre neoplastic disease while simultaneously decreasing over diagnosis of benign processes. [4]Many studies have shown that with proper training, MLBC results in a better diagnostic yield than traditional cervical smears [11, 14, 15].

Many dysplasia-associated biomarkers have been identified which are being employed to improve the diagnostic accuracy of neoplastic and pre-neoplastic lesions of the cervix histologically and cytological. [16,17] Human papilloma virus (HPV) infection is an imperative cause for the progress of cervical cancers. P16 expression, which can be detected immunohistochemically, is directly related to the presence of HPV. Thus, this protein can be used as a biomarker that can add significant diagnostic precision in the assessment of CIN lesions [18]. p16INK4A (p16) and Ki-67 are two representative markers of high-risk HPV infection and high-grade Cervical Intraepithelial Neoplasia. [19]

Through this study we aim at establishing that MLBC with p16 immunomarker is very effective in screening for cervical cancer as it overcomes the major setbacks like inadequate sampling, drying artefacts and obscuring blood which are seen in conventional PAP smear technique.

## REVIEW OF LITERATURE

According to World Health Organization,

Cancer is a generic term for a large group of diseases characterized by the growth of abnormal cells beyond their usual boundaries that can then invade adjoining parts of the body and/or spread to other organs.

Human papillomavirus (HPV) is an extremely common group of viruses in the world, which is known to cause cervical carcinoma. Out of the more than 100 types of HPV identified, about 14 are carcinogenic or high risk type. Cervical cancer is transmitted as a sexually acquired infection with certain types of HPV.HPV 16 and 18 are the high risk types responsible for 70% of cervical cancers and pre-cancerous cervical lesions.[20]

Cervical cancer is recognized as a rare long-term outcome of persistent infection of the lower genital tract by one of about 15 high-risk HPV types, which is termed the “necessary” cause of cervical cancer. Of the estimated 530 000 new cervical cancer cases annually, HPV 16 and HPV 18 account for 71% of cases; while HPV types 31, 33, 45, 52, and 58 are responsible for another 19% of cervical cancer cases**.** It has been recorded that nearly 90% of incident HPV infections are not detectable within a period of 2 years from the acquisition of infection and persist only in a small part of the affected population. [20]

The tests used widely to diagnose cervical malignant and premalignant conditions include conventional cytology (Pap smear), in recent years liquid-based cytology and HPV testing, and, in LMICs, visual inspection with acetic acid (VIA).[21] While the Pap smear is still the major backbone of screening and is related with substantial reduction in cervical cancer risk in high-income countries, it is a challenging and resource intensive technology that is not affordable in many low-resource settings[21]

According to Bobdey S et al, Cervical cancer is a significant public health problem in developing countries like India, such that India alone accounts for one-quarter of the worldwide burden of cervical cancers which is not quite the case in the developed countries of the world.[22,23] It is the one of the leading cause of deaths due to cancer, accounting for 17% of all cancer deaths among women aged between 30 and 69 years. 1 in 53 Indian women are likely to develop cervical carcinoma during their lifetime as opposed to 1 in 100 women in more developed regions of the world.[23] After exploring various diagnostic techniques for detection of cervical carcinoma, the pooled estimates of sensitivity and specificity of visual inspection with acetic acid (VIA), magnified VIA, visual inspection with Lugol's iodine (VILI), cytology (Pap smear), and human papillomavirus DNA were found to be 67.65% and 84.32%, 65.36% and 85.76%, 78.27% and 87.10%, 62.11% and 93.51%, and 77.81% and 91.54%, respectively.[24]

Dr. Aruna Rastogi has stated in her article on Cervical cancer published in National Health Portal that, every year in India, 122,844 women are diagnosed with cervical cancer and 67,477 die from the disease. [25]

| Stage | Description |
|---|---|
| I | The carcinoma is strictly confined to the cervix (extension to the uterine corpus should be disregarded). |
| **IA** | Invasive carcinoma that can be diagnosed only by microscopy, with maximum depth of invasion <5 mm |
| **IA1** | Measured stromal invasion <3 mm in depth |
| **IA2** | Measured stromal invasion ≥3 mm and <5 mm in depth |
| **IB** | Invasive carcinoma with measured deepest invasion ≥5 mm (greater than Stage IA), lesion limited to the cervix uteri |
| **IB1** | Invasive carcinoma ≥5 mm depth of stromal invasion, and <2 cm in greatest dimension |
| **IB2** | Invasive carcinoma ≥2 cm and <4 cm in greatest dimension |
| **IB3** | Invasive carcinoma ≥4 cm in greatest dimension |
| **II** | The carcinoma invades beyond the uterus, but has not extended onto the lower third of the vagina or to the pelvic wall |
| **IIA** | Involvement limited to the upper two-thirds of the vagina without parametrial involvement |
| **IIA1** | Invasive carcinoma <4 cm in greatest dimension |
| **IIA2** | Invasive carcinoma ≥4 cm in greatest dimension |
| **IIB** | With parametrial involvement but not up to the pelvic wall |
| **III** | The carcinoma involves the lower third of the vagina and/or extends to the pelvic wall and/or causes hydronephrosis or nonfunctioning kidney and/or involves pelvic and/or para-aortic lymph nodes |
| **IIIA** | The carcinoma involves the lower third of the vagina, with no extension to the pelvic wall |
| **IIIB** | Extension to the pelvic wall and/or hydronephrosis or nonfunctioning kidney (unless known to be due to another cause) |
| **IIIC** | Involvement of pelvic and/or para-aortic lymph nodes, irrespective of tumor size and extent |
| **IIIC1** | Pelvic lymph node metastasis only |
| **IIIC2** | Para-aortic lymph node metastasis |
| **IV** | The carcinoma has extended beyond the true pelvis or has involved (biopsy proven) the mucosa of the bladder or rectum. |
| **IVA** | Spread to adjacent pelvic organs |
| **IVB** | Spread to distant organs |

**TABLE 1**: FIGO CLASSIFICATION OF STAGES OF CERVICAL CARCINOMA

[26]The table has been taken from Cervix Cancer Facts by Rajiv Gandhi Cancer Institute & Research Centre, New Delhi.

According to the works of Sreedevi A et al, Published in Int J Womens Health, Age adjusted incidence rates of cervix uteri-females (rate per 100,000) in the various population based cancer registries are as follows.

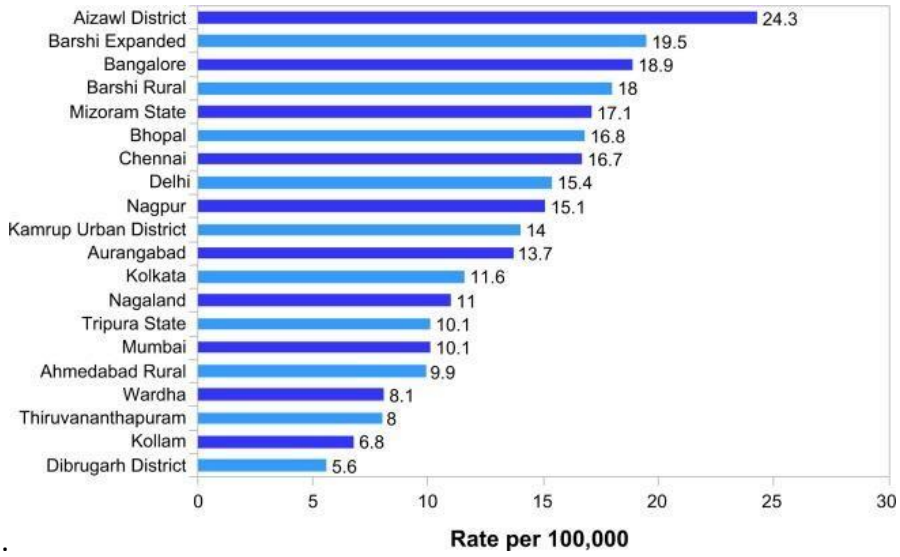


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**Figure -1** Age adjusted incidence rates of cervix uteri-females (rate per 100,000)

We can thus infer from Figure -1,that the highest age-adjusted rates are in Aizawl in the north eastern part of India at 24.3 per 100,000 women[27]

International Comparisons of Age Adjusted Incidence Rates with that of PBCRs under NCRP shows CERVIX UTERI (ICD-10 : C53) – Females as mentioned in [28]

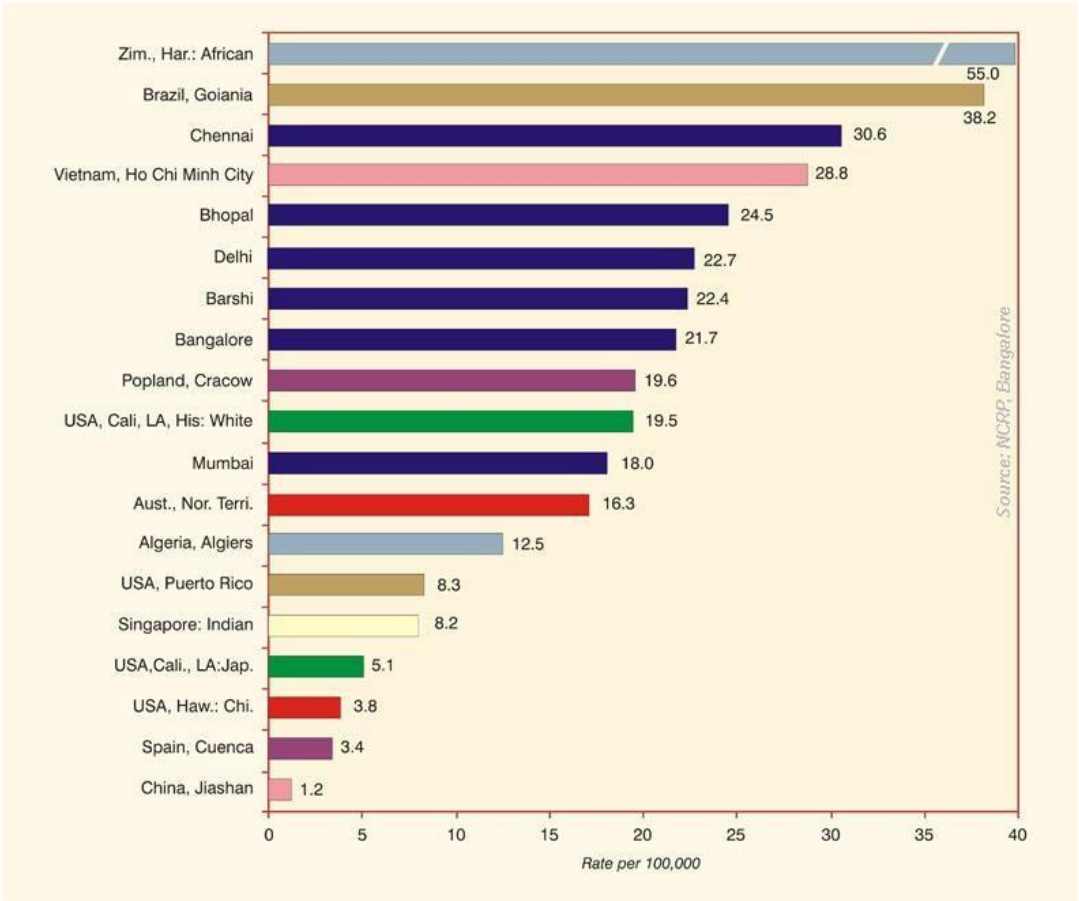



**Figure -2:** International Comparisons of Age Adjusted Incidence Rates with that of PBCRs under NCRP

**TABLE-2:** TOTAL COST INCURRED WHILE IMPLEMENTING VARIOUS SCREENING STRATEGIES

| Screening strategy | | Screening cost in million | | Treatment expenditure in million | | Total cost in million* | |
|---|---|---|---|---|---|---|---|
| | | INR | USD | INR | USD | INR | USD |
| No organized Screening | | 19 (11-32) | 0.29 (0.17-0.48) | 175 (103-291) | 2.65 (1.56-4.40) | 194 (114-323) | 2.93 (1.72-4.88) |
| Visual inspection with acetic acid | 3 Years | 583 (429-757) | 8.81 (6.49-11.44) | 119 (88-155) | 1.80 (1.33-2.34) | 702(517-912) | 10.61 (7.82-13.79) |
| | 5 Years | 315 (236-400) | 4.76 (3.57-6.05) | 128 (96-164) | 1.93 (1.45-2.48) | 443(332-564) | 6.70 (5.02-8.53) |
| | 10 Years | 190 (140-251) | 2.87 (2.12-3.79) | 137 (102-181) | 2.07 (1.54-2.74) | 327(242-432) | 4.94 (3.66-6.53) |
| PAP smear | 3 Years | 633 (449-836) | 9.57 (6.79-12.64) | 121 (86-159) | 1.83 (1.30-2.40) | 754(535-995) | 11.40 (8.09-15.04) |
| | 5 Years | 348 (250-459) | 5.26 (3.78-6.94) | 136 (97-179) | 2.06 (1.47-2.71) | 484(347-638) | 7.32 (5.25-9.64) |
| | 10 Years | 209 (152-278) | 3.16 (2.30-4.20) | 139 (101-185) | 2.10 (1.53-2.80) | 348(253-463) | 5.26 (3.82-7.00) |
| HPV DNA test | 3 Years | 837 (625-1155) | 12.65 (9.45-17.46) | 114 (85-157) | 1.72 (1.28-2.37) | 951(710-1312) | 14.38 (10.73-19.83) |
| | 5 Years | 472 (352-647) | 7.14 (5.32-9.78) | 125 (93-172) | 1.89 (1.41-2.60) | 597(445-819) | 9.02 (6.73-12.38) |
| | 10 Years | 284 (211-386) | 4.29 (3.19-5.84) | 133 (99-181) | 2.01 (1.50-2.74) | 417(310-567) | 6.30 (4.69-8.57) |

* Total cost in a cohort of 1 lakh population; Pap: Papanicolaou test; Values in parenthesis represent 2.5$^{th}$ and 97.5$^{th}$ percentile

Picture courtesy (29)

The analysis of the Table-2 states that when applied over a longer duration of time , the cost of the screening techniques shows an overall decrease, providing more specific and sensitive tests without causing a significant economical imbalance. Hence a long term investment by the government in the direction of diagnostic techniques for cervical carcinoma is sure to ensure early detection and better prognosis of the condition.

**TABLE-3:** COST OF CERVICAL CANCER SCREENING IN INDIA AS REPORTED IN VARIOUS STUDIES

| Parameter | Present study | Diaz et al (2008), Goldie et al (2005) | | Legood et al (2005) | |
|---|---|---|---|---|---|
| | INR (2017) | I$ 2005 | Converted to INR 2017 | US$ 2005 | Converted to INR 2017 |
| Cost per woman screened with VIA test | 344 | 1.25 | 32.21 | 3.917 | 396.94 |
| Cost per woman screened with Cytology test | 652 | 3.69 | 96.11 | 6.609 | 773.88 |
| Cost per woman screened with HPV DNA test | 980 | 10.30 | 265.73 | 11.779 | 1404.49 |

Picture courtesy [29]

This analysis (as per table 3) on cervical cancer screening in India reveals that, cost per woman for screening for cervical carcinoma is maximum with HPV DNA test , while the least with VIA test, Cytological testing being intermediate amounting to about INR 652 according to the most recent study undertaken when report[29] was made.

In the study Conventional Pap smear and liquid based cytology for cervical cancer screening - a comparative study, by Sherwani et al, Infections agents were detected in 14 (8.7%) cases on (Liquid based cytology ) Pap spin and in 5 (3.1%) cases on conventional Pap smear. Candida being the commonest infectious agent in (4.3%) cases, followed *by Trichomonas vaginalis* which were detected majorly on liquid based cytology smears.[11]

A comparative study of Pap spin(Liquid based cytology ) and histo-pathologocial findings performed. Out of the 29 cases diagnosed as LSIL on Pap spin , 2 (6.9%) case each had normal histology and moderate dysplasia and 26 (89.7%) cases had mild dysplastic changes on histopathology. Out of the 7 cases of HSIL 1 (14.3%) had mild dysplasia and 6 (85.7%) cases had moderate dysplasia and all the 6 cases of carcinoma on Pap spin revealed squamous cell carcinoma on histopathology.[11]

A similar comparative study between findings on conventional Pap smear and histopathology revealed that out of 17 cases diagnosed as LSIL on conventional Pap smear, 2 each (11.8%) had normal histology and moderate dysplasia whereas 13 cases (76.5%) had mild dysplasia on histopathological study. A single case of HSIL on Pap smear was diagnosed as moderate dysplasia on histopathology, whereas all the 6 cases of carcinoma on Pap smear were diagnosed as squamous cell carcinoma (large cell non keratinizing type) on histopathology.[11]

Kavatkar et al have, in their article on Study of a manual method of liquid-based cervical cytology mentioned liquid-based cervical cytology as a technique which enables cells to be suspended in a monolayer and thus enabling better morphological assessment . It includes the preparation and evaluation of cells collected in a liquid fixative. It is being introduced in developed countries to improve the sensitivity of the Pap test. The advantages of liquid-based cytology involves improved sensitivity and specificity because of better fixation of cells and well preserved nuclear details. Abnormal cells do not get obscured or diluted by other epithelial or inflammatory cells. There is, therefore, a lower rate of unsatisfactory cervical cytology samples. The residual cell suspension can be used to make further cytological preparations or used for other tests like detection of human papilloma virus (HPV) DNA. Other ancillary techniques like immunocytochemistry which includes using various immune markers can also be performed on the residual sample.[13]

The more widely used technologies for liquid-based cytology demand expensive equipments. They have , in order to reduce the cost factor involved in detection, used a manual membrane-based method using simple equipment - a vortexer and laboratory centrifuge. Slides prepared

by using a polymer solution were allowed to dry, forming a membrane.[13]

Quoting Kalof and Cooper's study, Yu et al have mentioned p16 as a cell-cycle inhibitor which binds to cyclin-dependent kinase 4 or cyclin dependent kinase 6 (CDK4)/CDK6, preventing the phosphorylation and subsequent inactivation of the retinoblastoma protein (pRB).[30,31]

The down-regulation of p16 has been linked with carcinogenesis in many organ systems.[32,33] Overexpression of viral proteins E6 and E7[34]; E7, in turn, binds to and inactivates pRB and induces the production of p16 through a negative occurs as a result of integration of HPV DNA into the host genome forming the feedback loop.[35] Ki-67 is another marker correlated with the grade of dysplastic variations in the cervical epithelium which Yu et al have explored in their study (Diagnostic value of p16INK4A, Ki-67, and human papillomavirus L1 capsid protein immunochemical staining on cell blocks from residual liquid-based gynecologic cytology specimens). Positive immunostaining for both p16 and Ki-67 in the upper two-thirds of the squamous epithelium is indicative of neoplastic atypical squamous lesions.[36]

According to the study by Mao et al , Pap smear has been an effective screening tool for cervical cancer, however it's sensitivity limited with fairly high cost and need for significant infrastructure and technology to be effective.[37] Human papillomavirus (HPV) testing has been proposed as a possible screening tool in resource poor settings where Pap screening is not feasible; however testing for high-risk HPV deoxyribonucleic acid (DNA) is limited by its poor specificity.[38] The hunt for specific biomarkers of HPV infected premalignant cells has yielded a number of possible markers of disease, including $p16^{INK4a}$.[37]

$p16^{INK4a}$ protein participates in cell cycle regulation and accumulates in abnormal epithelial cells infected with high-risk Human Papilloma Virus. $p16^{INK4a}$ is a cyclin dependent kinase (CDK) inhibitor regulating the activity of CDK4 and CDK6. Its inactivation by hyper methylation is a common occurrence in carcinomas.[39]In non-HPV linked tumours, this inactivation leads to increased CDK functioning and inactivation of the retinoblastoma protein (pRb), resulting in cell cycle disruption and increased proliferation of cell. However, in HPV-associated tumours, inactivation of pRb by high-risk HPV oncoprotein E7 leads to cell proliferation and markedly increased levels of $p16^{INK4a}$. Immunostaining of histology and cytology specimens for $p16^{INK4a}$ has revealed that detection of antibodies to the protein is a sensitive and specific marker of high-grade cervical intraepithelial neoplasia (CIN) and invasive cervical cancer.[39,40,41,42] Many studies have reported $p16^{INK4a}$ staining of biopsy tissue as being generally absent from the normal tissue, showing only zonal or patchy staining of the low-grade CIN cases and present in over 90% of cases with high-grade CIN and invasive cervical cancer,.[39,43,44] Findings from these immunostaining studies conducted by suggest that

compared to high-risk HPV DNA, which is detected in the majority of both high- and low-grade lesions,[45] $p16^{INK4a}$ might be a better marker for identifying those lesions most likely to progress to malignancy.[46]

From their study on Diagnostic Value of p16INK4A, Ki-67, and Human Papillomavirus L1 Capsid Protein Immunochemical Staining on Cell Blocks From Residual Liquid-Based Gynecologic Cytology Specimens, Yu et al reported greater immunoreactivity for p16 and Ki-67 in HSIL and SCC specimens in cell block sections, which were in accordance with the results from histopathology samples in their previous study , Expression of p16INK4A and Ki-67 in cervical intraepithelial neoplasia published in J Clin Exp Pathol. 2007; 23:665-668 and suggesting that p16 and Ki-67 immunostaining on a cell block could improve the accuracy of detecting HSIL and SCC. They discovered that intensity of p16 staining on a cell block also is a significant index for the determination of high-grade CIN. Thus, strong staining for p16 was observed in the cell block preparations from all SCC sections. Strong, full-thickness epithelial staining being documented only in the HSIL and SCC specimens, can serve as a valuable indicator of high-grade cervical lesions.29,30 Either weak staining or cells less positive to p16 were noted in the cell block preparations from low-grade cervical intra epithelial lesion or LSIL.[31]

## OBJECTIVES OF THE STUDY

**Primary Objective:** To improve methods of diagnosis of malignant and pre malignant lesions of the cervix.

**Secondary Objectives:**

1) To screen the obtained cervical samples using Conventional Pap Smear (CPS) for presence of malignant or pre malignant squamous cells,

2) To study the residual samples obtained by the Ayres spatula using Manual Liquid Based Cytology (MLBC) and access for presence of malignant or pre malignant squamous cells,

3) To compare the CPS and MLBC findings and confirm by biopsy wherever possible.

4) To use p16immunomarker an ancillary technique on MLBC and study its use in improving diagnostic accuracy.

## MATERIALS AND METHODOLOGY:

**Methods:**

Conventional Pap smear

Manual Liquid Based Cytology with Immunomarker p16 on the samples with cytomorphological changes.

**Materials:**

**Chemicals-**

10% formalin for fixation
Haematoxylin and Eosin stain
Papanicolaou stain
Sodium chloride, sodium citrate,95% alcohol
P16ink4a as primary antibody

**Instruments-**

Plastic or wooden Ayre's spatula
Cytobrush
REMI R-8C laboratory Centrifugation Machine
Normal glass slides
Pressure cooker or microwave
Refrigerator
Incubator
Compound Microscope

**Method of data collection**

Written informed consent will be obtained from all patients involved in the study

**Methodology**

Procedure-

1) Samples were collected by 360 degree rotation of cytobrush in the transformation zone of the cervix.
2) Then the sample was transferred onto a clean glass slide and stained with conventional Pap stain.
3)Another rotation was taken and that sample was transferred to 5 ml of liquid fixative(5 gm of sodium chloride ,5gm of sodium citrate,50 ml of 10%formalin and 50ml of 95% alcohol) kept overnight and centrifuged at 800 rpm for 10 minutes.
4) Fixative was decanted and the excess fixative was blotted.
5) 1-2ml of polymer solution (contains agarose, polyethylene glycol, alcohol and poly-lysine) was added to tube.
6) Vortex mixing was done
7) 3-6 drops of suspension were applied to the glass slide
8) Allowed to dry

9) It was stained with conventional Pap stain.

10) The Bethesda system for reporting cervical cytology was used.

11) All the samples were collected using the above mentioned procedure, those which showed cytomorphological changes were used for application of p16 immunomarker on the smear slide itself (application of the primary and secondary antibodies)

12) Scoring was done as following: Negative (no staining or <3 positively stained cells), 1+ (3-10 positively stained cells), 2+ (>10 positively stained cells). Along with the cell number, staining intensity was also taken into consideration as mentioned in [6]

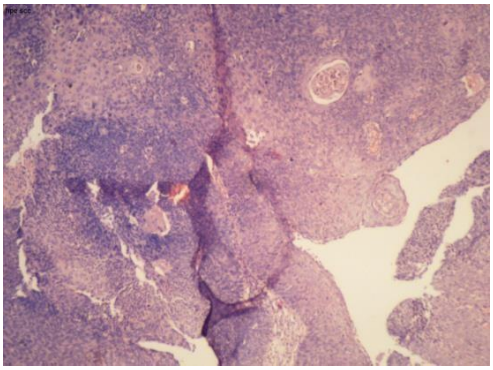


Figure 3

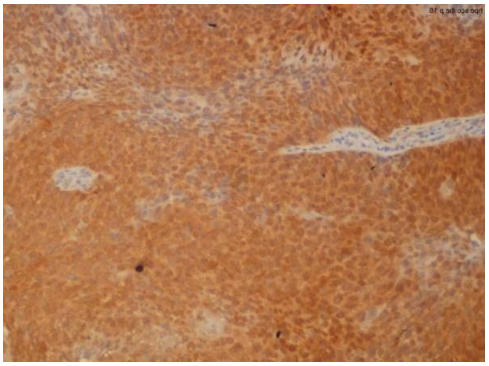
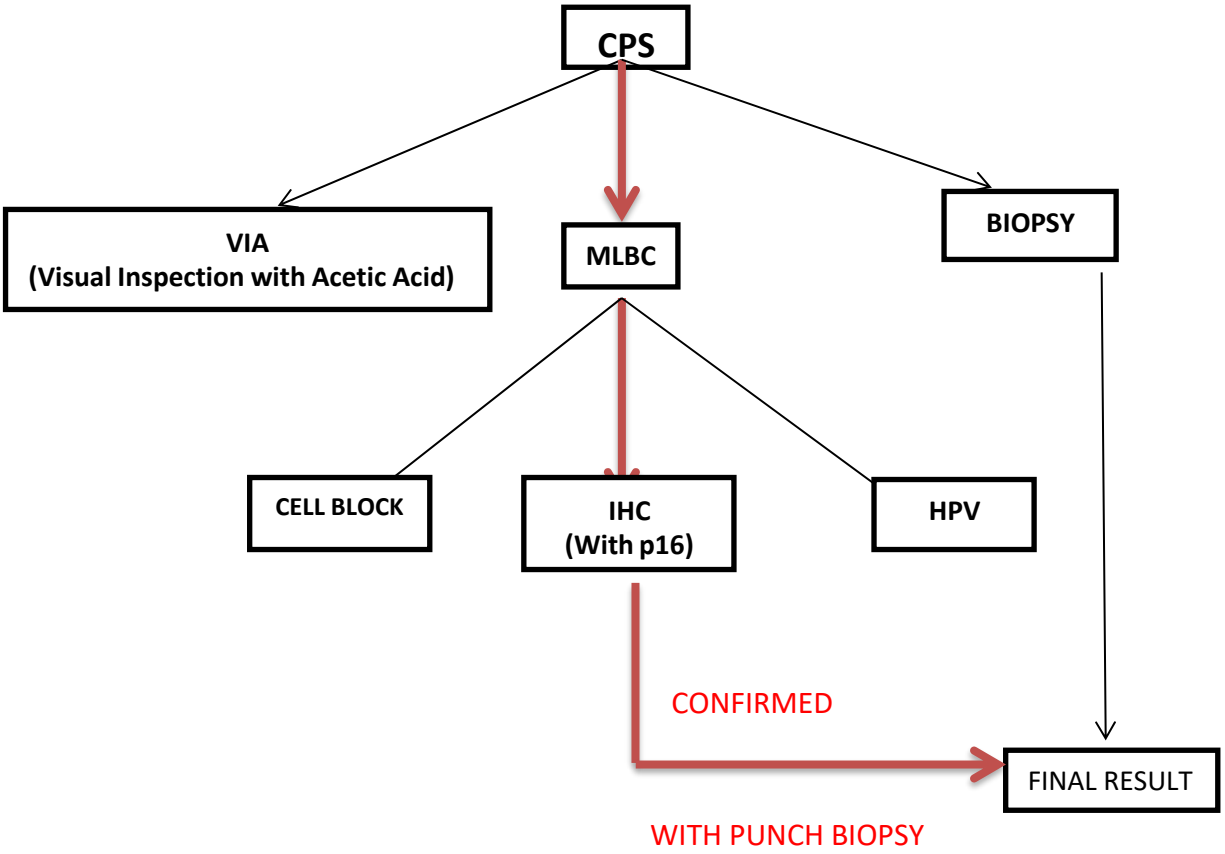


Figure 4

Figure 3 Squamous cell carcinoma cervix with Haematoxylin and Eosin (H&E) staining
Figure 4 Squamous cell carcinoma cervix with p16 marker strongly positive
Courtesy- Department of Pathology, JSS Medical College, Mysuru.

**Figure 5:**Various available screening modalities and the method followed (highlighted in red)




**STUDY DESIGN:** Quasi experimental

**SUBJECT ELIGIBILITY**

**Inclusion criteria:**

Women aged 21 and above
Women with family history of cervical cancer
Women with symptoms indicative of cervical cancer like excessive white discharge, pain abdomen, dysmenorrhoea, dyspareunia, bleeding per vagina and genital ulcers

**Exclusion criteria**

The women who were not willing to be a part of the study.

The women who had been screened previously and had been diagnosed with cervical cancer.
The women who had undergone hysterectomy with removal of cervix

**Study place:** Mysuru

**Study duration:** 2 months of data collection and 1 month of data analysis

**Sampling technique:** Purpose Sampling

**Study subjects:**

Women who were undergoing screening for cervical cancer (with complaints of white discharge, bleeding per vagina, pain abdomen, dysmenorrhoea, dyspareunia and genital ulcers)

**OBSERVATION AND RESULT**

According to The Mayo Clinic, the routine PAP Smear test should be done once in 3 years in women after they are sexually active (21 to 65 years of age).In our study , we saw patients aged from 21 years to 56 years .

An analysis of the patients screened with respect to their complaints is as follows;

**TABLE 4: WHITE DISCHARGE PER VAGINUM**

| | **Frequency** | **Percentage (%)** |
|---|---|---|
| Present | 15 | 30 |
| Absent | 35 | 70 |
| Total | 50 | 100 |

White discharge per vaginum was the major complaint accounting up to 30 %( 15 cases) out of total of 50 cases.

**TABLE 5: BLEEDING PER VAGINUM**

| | **Frequency** | **Percentage (%)** |
|---|---|---|
| Present | 5 | 10 |
| Absent | 45 | 90 |
| Total | 50 | 100 |

The study revealed about 10% of the women who got screened for cervical intraepithelial lesions had a history of bleeding per vaginum.

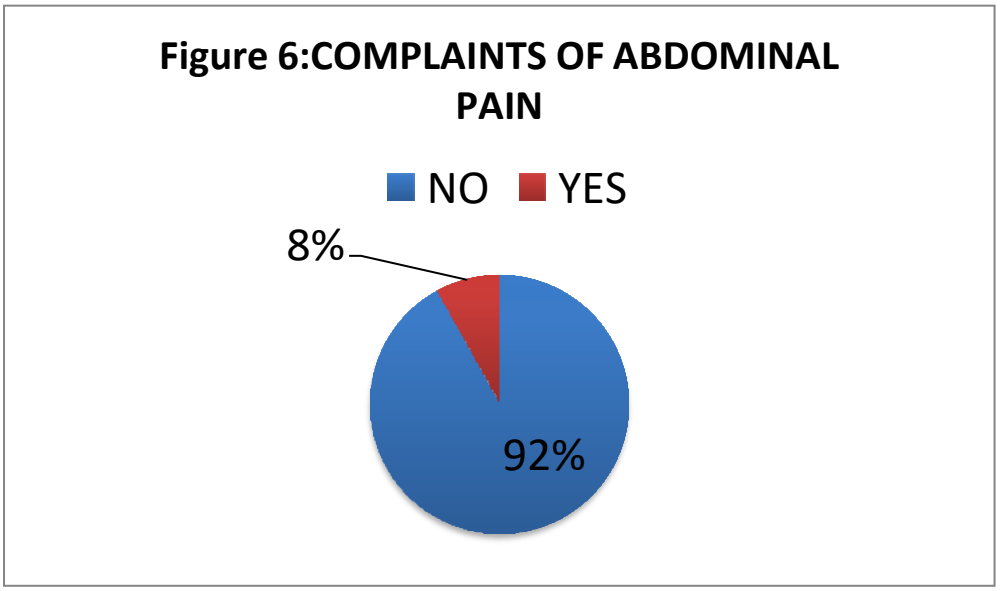
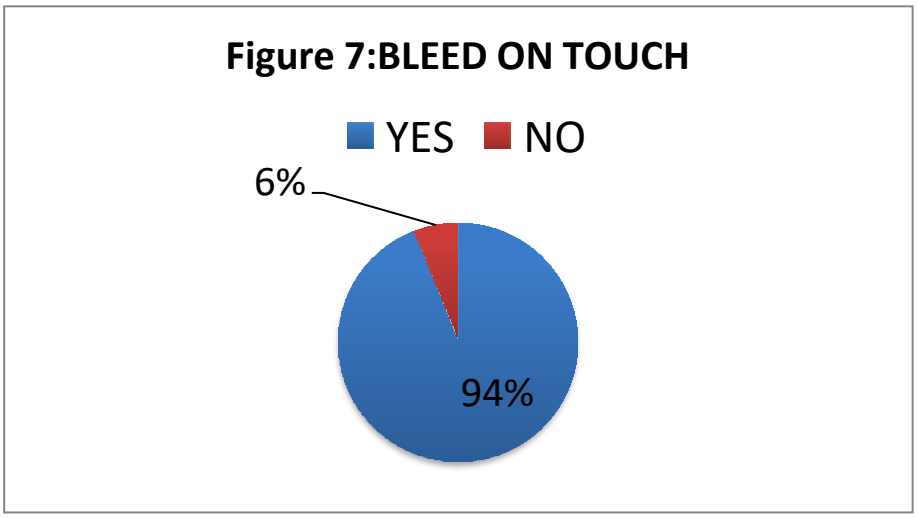



Figure 6 explains that in our study, we observed 4(8%) patients with complaints of abdominal pain, who got screened for cervical epithelial pathologies.

**TABLE 6: CERVICAL EROSION**

| | Frequency | Percentage (%) |
|---|---|---|
| Present | 4 | 8 |
| Absent | 46 | 92 |
| Total | 50 | 100 |

Table 6 states that Cervical erosions were seen in per speculum examination in 4 (8%) of the patients who were screened .




According to figure 7-Only 6% of the screened women complained of bleeding on touch. It is a classical symptom of cervical intraepithelial lesions.

**TABLE 7: COMPLAINTS OF POST MENOPAUSAL BLEEDING**

| | Frequency | Percentage (%) |
|---|---|---|
| Present | 3 | 6 |
| Absent | 47 | 94 |
| Total | 50 | 100 |

According to Table 7, 3(6%) of women who were screened complained of post-menopausal bleeding

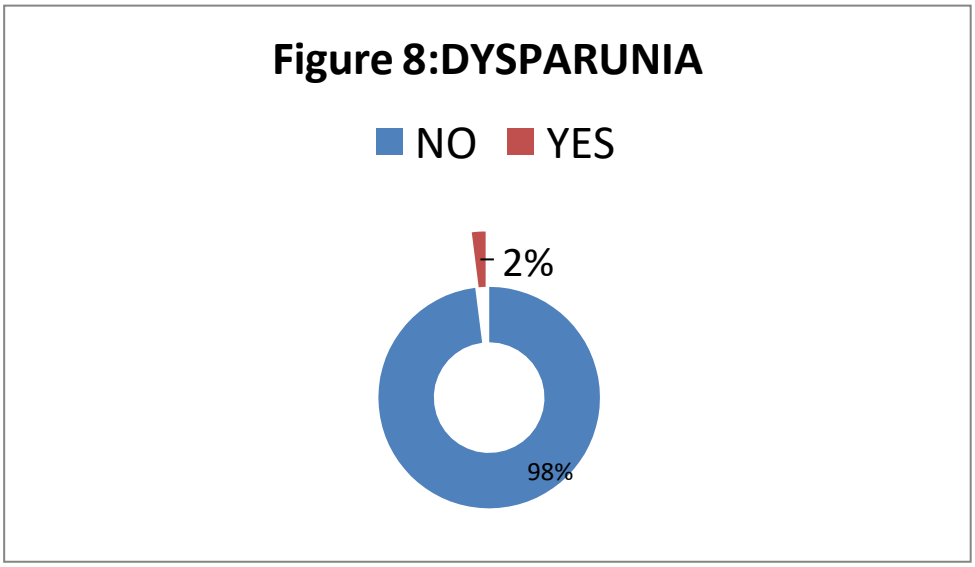



Figure 8 states that dysparunia or pain during coitus was a complaint of only 1(2%) of the ladies who were screened for cervical lesions.

We also found 5 normal smears which were those of women who came for routine screening or with conditions that were not directly linked to development of cervical lesions like oligomenorrhoea and primary infertility.

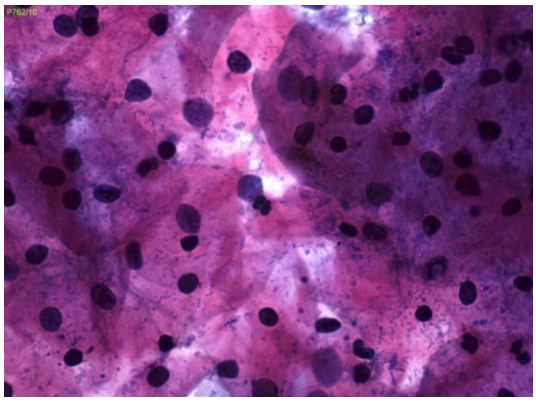


Figure 9

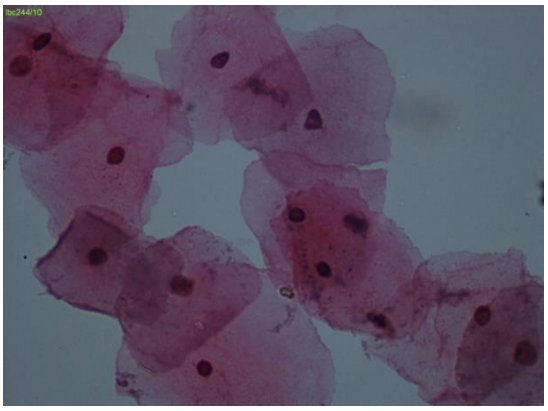


Figure 10

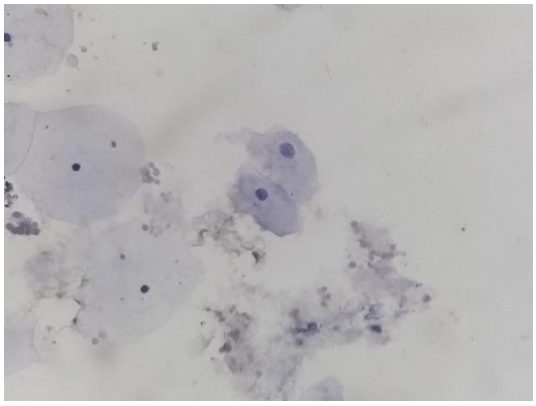


Figure 11

Figure 9: Normal smear as seen in PAP smear

Figure 10:Normal smear as seen in MLBC

Figure 11:Normal smear stains negative for p16 marker on MLBC slide.

PAP smear shows superficial and intermediate squamous cells of normal morphology, however overlapping of cells is seen.(FIGURE 9)

While MLBC has a monolayer of cells owing to the poly l-lysine used in the MLBC fixative that was prepared in the laboratory in the Department of Pathology, JSSH , Mysuru.( FIGURE 10)

The p16 marker doesn't stain the normal smear as it attaches only to the nuclei of cells which have been infected by high risk strains of HPV.( FIGURE 11)

**TABLE 8: AN ANALYSIS OF SYNCHRONY IN RESULTS OBTAINED BY CPS AND MLBC**

| | | | MLBC+P16 | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | **NILM** | **Koilocytic Atypia** | **LSIL** | **HSIL** | **SCC** | **Bacterial Vaginosis** | **Inadequ-ate smear** | **Total** |
| **CPS** | **Pre malignant** | Count | 26 | 1 | 2 | 0 | 0 | 3 | 3 | 35 |
| | | % within CPS | 74.3% | 2.9% | 5.7% | .0% | .0% | 8.6% | 8.6% | 100.0% |
| | **Malignant** | Count | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 2 |
| | | % within CPS | .0% | .0% | .0% | 50.0% | 50.0% | .0% | .0% | 100.0% |
| **Total** | | Count | 26 | 1 | 2 | 1 | 1 | 3 | 3 | 37 |
| | | % within CPS | 70.3% | 2.7% | 5.4% | 2.7% | 2.7% | 8.1% | 8.1% | 100.0% |

Under premalignant conditions, we have considered NILM (Negative for Intraepithelial Lesion or Malignancy), Koilocytic atypia and LSIL (Low grade Squamous Intraepithelial Lesion).
The malignant conditions include HSIL (High grade Squamous Intraepithelial Lesion) and SCC (Squamous Cell Carcinoma).

From Table 8, we infer that out of the total of 37 cases which were observed to be pre malignant or malignant, we reported 2 cases as malignant cases namely the HSIL and SCC , one case in each scenario were reported by both CPS and MLBC.The reports were affirmative on application of p16 marker on MLBC slide also.

There was a 74.3%(26 cases) correlation between those cases considered as premalignant according to CPS and NILM according to p16 marker run on MLBC slides.P16 doesn't stain brown in NILM conditions.

Similarly 1 case(2.9%) of the premalignant conditions(CPS) were reported as having koilocytic atypia ( MLBC).

Two of the premalignant cases were found to be LSIL by MLBC analysis,also by application of p16 marker which doesn't stain brown.

There were also 3 cases found to be premalignant according to CPS as bacterial vaginosis, owing to the clearer background and monolayer suspension of the cells seen in MLBC.

Also 3 ( MLBC) smears were inadequate, which implies that a good level of experience and accuracy in processing of the MLBC slides is essential to get correct diagnosis.

**NEGATIVE FOR INTRAEPITHELIAL LESION OR MALIGNANCY (NILM)**

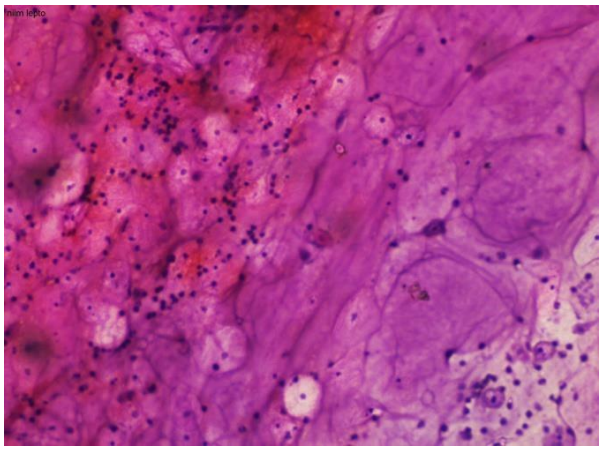


**Figure 12:**NILM by CPS

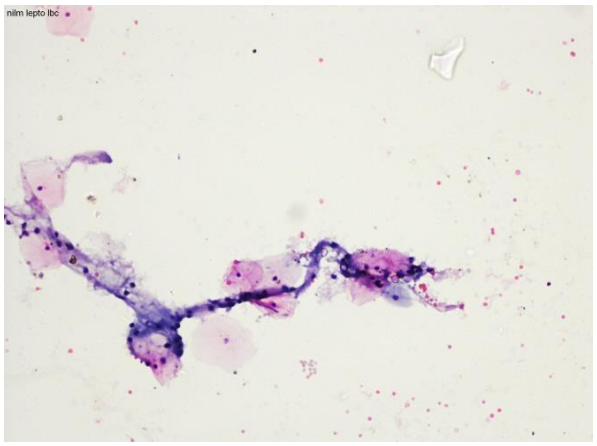



**Figure 13:**NILM showing presence of leptothrix(commensal) by MLBC

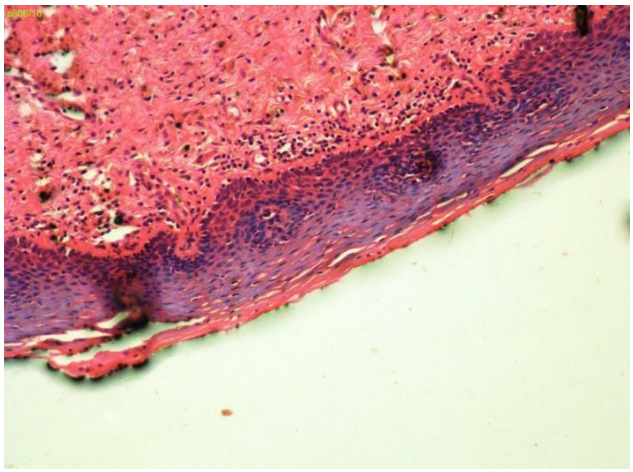
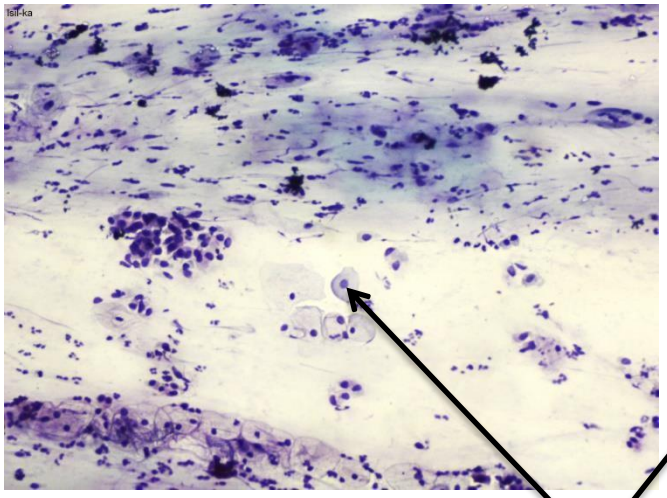


**Figure 14:**NILM in histopathological section

NILM slide of the same case analysed by using both CPS(figure 12) and MLBC(figure 13) shows presence of the inflammatory cells and also leptothrix a commensal bacterium found in the vagina normally, which is not that appreciable in slide made by using CPS, multiple layers being the major obstructing factors.

**LOW GRADE SQUAMOUS INTRAEPITHELIAL LESION (LSIL)**



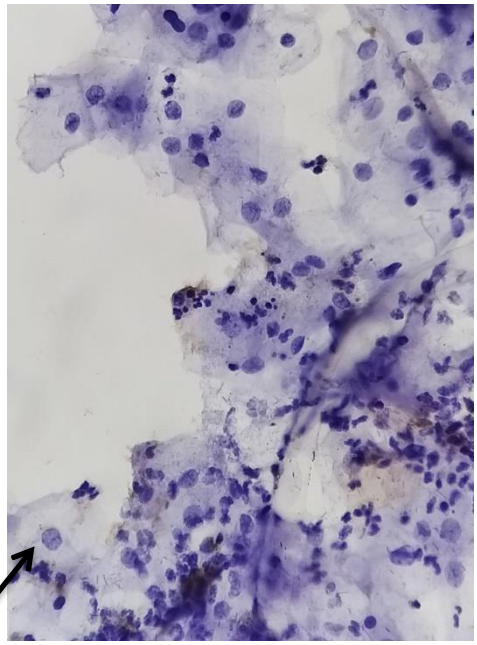



**Figure 15** Koilocyte **Figure 16**

**Figure 15**:LSIL by CPS

**Figure 16**: LSIL by P16 on MLBC is negative

P16 negative on LSIL in our study sample which is indicative that the infection is by a low risk strain of HPV and that it will not progress to malignancy(Figure 16).

Koilocytes, cells having large nuclei surrounded by peri-nuclear halo (characteristic of HPV infected superficial epithelial cells of the cervix) are seen in the smear made by CPS and MLBC. (Seen in both figure 15 and figure 16)

**HIGH GRADE SQUAMOUS INTRAEPITHELIAL LESION (HSIL)**

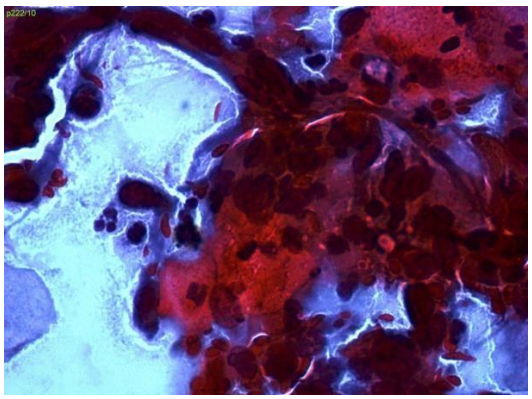


**Figure 17**

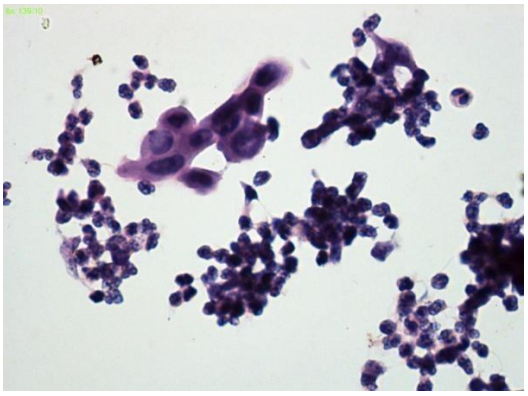


**Figure 18**

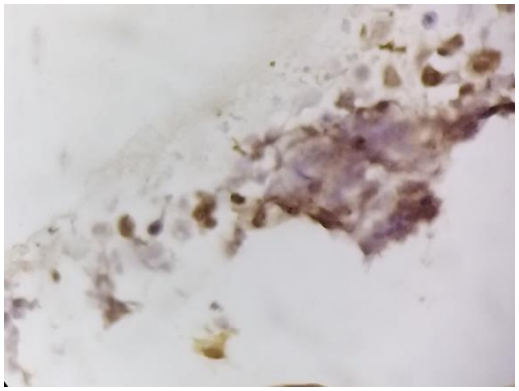


**Figure 19**

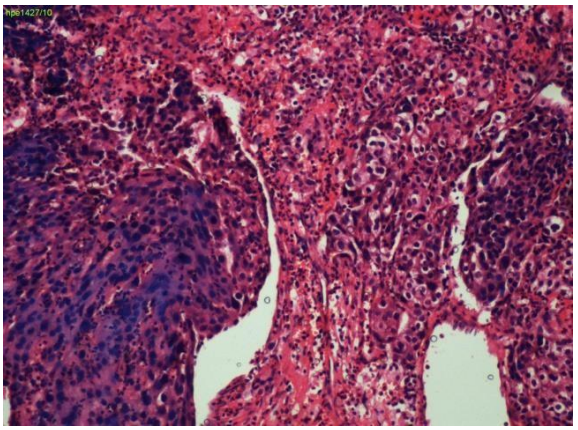


**Figure 20**

**Figure 17**:HSIL by CPS

**Figure 20::**HSIL by MLBC

**Figure 19**:HSIL stained positive by P16 on MLBC, implying that it is an infection by a high risk strain of HPV.

**Figure 20:** Histopathological or biopsy appearance of HSIL

HSIL is a crucial stage , which can have reactive atypia as a differential diagnosis.P16 marker is specific to those malignant cells which have been infected by the high risk strains of HPV like 16 and 18.Thereby if stained by p16 immunohistomarker, the patient needs to be treated for management of the carcinoma and if negative for p16 staining , then treatment for the underlying infectious or non malignant condition is sufficient

**SQUAMOUS CELL CARCINOMA (SCC)**

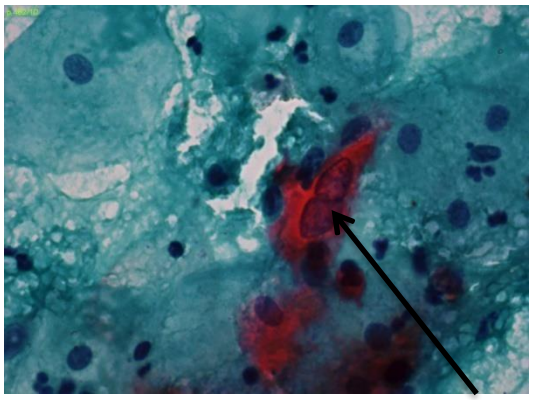


**Figure 21:**SCC BY CPS TADPOLE CELL

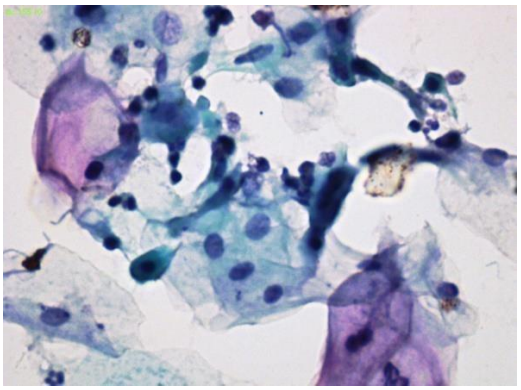


**Figure 22**: SCC BY MLBC

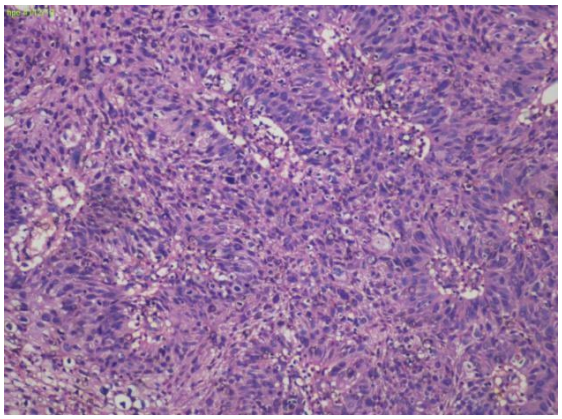


**Figure 23:**BIOPSY (histopathology) of SCC

Squamous cell carcinoma is characterised by appearance of tadpole cells appreciable in both CPS slide (figure 21) that invade into the deeper layers starting inwards from the squamous epithelial layer. It is highly invasive and can metastasize to the adjoining organs like uterus and lymph nodes and that demands radical treatment.

**INFECTIOUS CONDITIONS**

In our study, we observed two infectious conditions namely Bacterial Vaginosis and Trichomonas Vaginalis Infestation.

There were 10(20%) infectious conditions out of 50 cases that were subjected to screening by p16 marker on manual liquid based cytology smears. Out of these 10, 3 cases (6% of the total of 50 cases) were that of trichomonas infestation, the reports being affirmative for both CPS and P16 with MLBC.

However there were 7 cases diagnosed as Bacterial vaginalis according to our test method(p16 on MLBC) and CPS reported only 3 of them as Bacterial Vaginosis .

This can be attributed to the presence of obscuring factors like blood, inflammatory cells and non uniform as well as in multiple layers in CPS as opposed to a clean , monolayer of cells with minimal obscuring factors which ensures better visualization of the nuclear and the cytological morphological picture.

Of the 7 cases diagnosed as bacterial vaginosis, 3 women complained of white discharge per vaginum ,one out of them who also complained of greenish discharge per vaginum and post menopausal bleeding. It may be suggestive that lack of hygiene in post-menopausal period, can lead to infectious conditions which if not taken care of may lead to complications.

An important observation was that three of the seven bacterial vaginosis cases were diagnosed as NILM by CPS.

In 2 out of 3 (66.67%) cases diagnosed as Trichomonas Infestation , White discharge per Vaginum was reported.

The age of these patients ranged from 23 to 46 years implying that the development of this condition is linked with a callous attitude towards personal hygiene which is more prevalent among women in the thirties as per our study findings

**BACTERIAL VAGINOSIS**

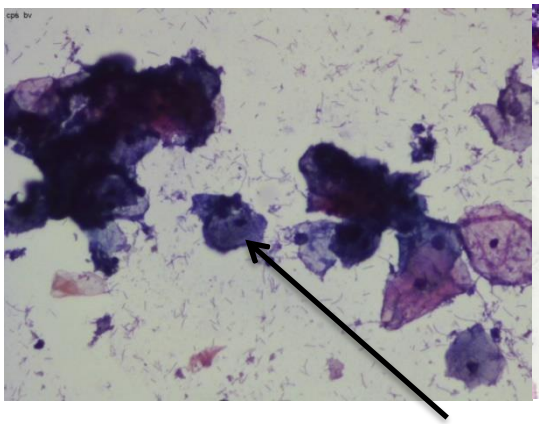



**Figure 24**

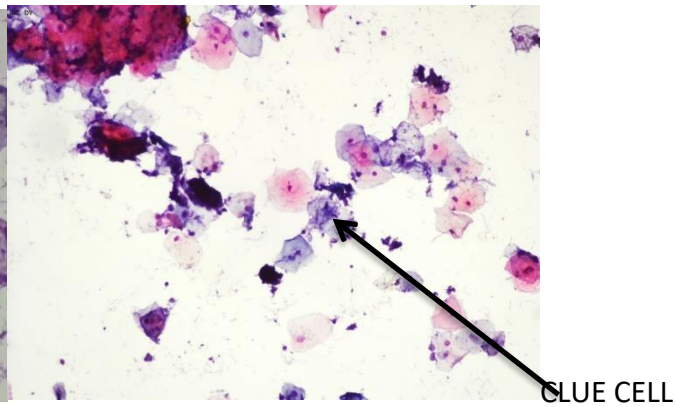



**Figure 25**

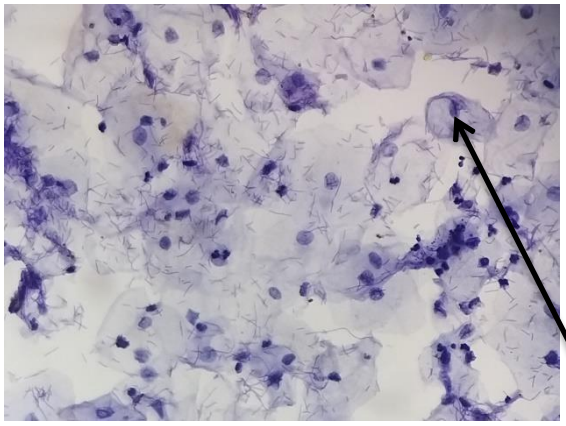



**Figure 26**

**Figure 24:** BACTERIAL VAGINOSIS BY CPS

**Figure 25:** BACTERISL VAGINOSIS BY MLBC

**Figure 26:**P16 ON MLBC IS NEGATIVE FOR BACTERIAL VAGINOSIS (NO BROWN STAINING SEEN IMPLYING IT CANNOT PROGRESS TO MALIGNANCY)

Clue cells (seen in figure 24, 25&26) are those cells which have dense cytoplasm infiltrated with non-commensal bacteria, more evident in MLBC against a clearer background as compared to Conventional PAP Smear.

**TRICHOMONIASIS**

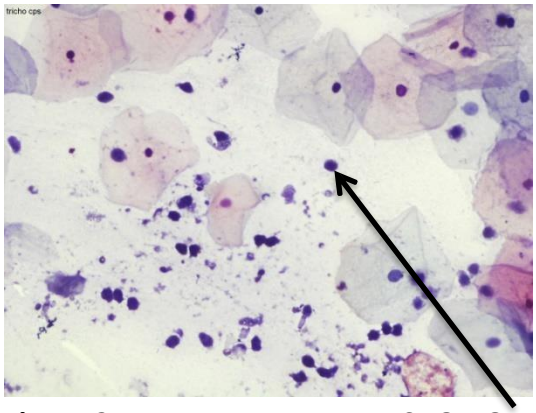



**Figure 27**

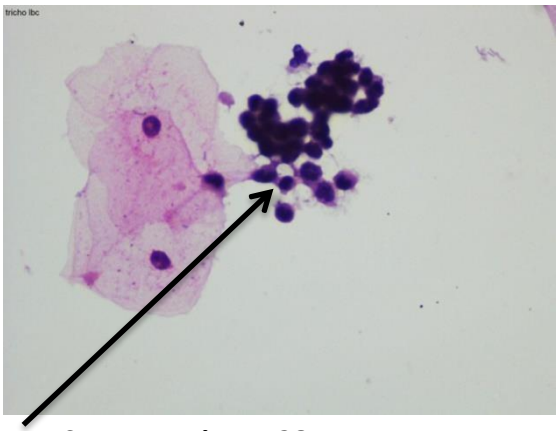



**Figure 28**

TRICHOMONAS VAGINALIS

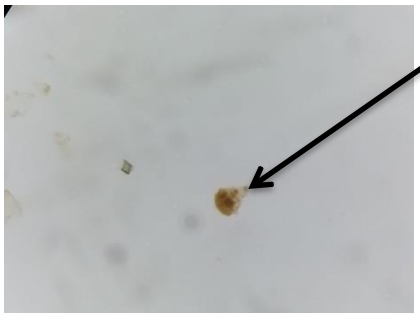


**Figure 29**

**Figure 27**:TRICHOMONIIASIS IN CPS

**Figure 28**:TRICHOMONIASIS IN MLBC

**Figure 29**: P16 on MLBC gives false positive in case of trichomoniasis.

The parasite Trichomonas Vaginalis is appreciable as a darkly stained ovoid structure outside the squamous epithelial cells in both CPS as well as in MLBC(FIGURE 27, 28). However the density of the parasites is more appreciable in MLBC because of the suspension of cells in a monolayer.

**ATROPHIC SMEARS**

While conducting the study, we also came across an atrophic smear , in which the senile changes resemble an inflammatory condition.

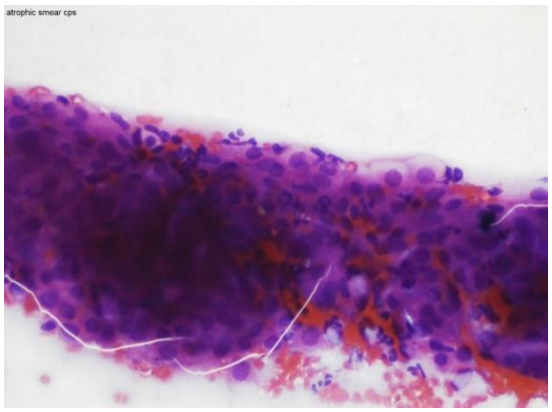



**Figure 30**: ATROPHIC SMEAR BY CPS

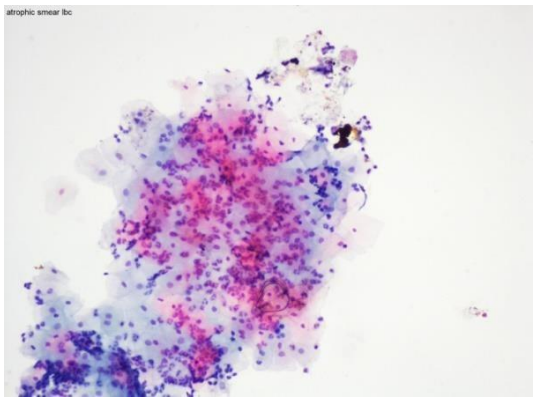



**Figure 31**:ATROPHIC SMEAR BY MLBC

**TABLE 9:** OVERALL ANALYSIS OF ALL THE PROCEDURES USED IN THE STUDY AND THE RESULTS OBTAINED

| **DIAGNOSIS** | **CPS** | **MLBC** | **MLBC +p16** | **HISTOPATHOLOGY** |
|---|---|---|---|---|
| NORMAL | 5 | 5 | - | 0 |
| NILM | 32 | 28 | - | 7 |
| Koilocytic Atypia | 1 | 1 | - | 0 |
| LSIL | 1 | 1 | - | 0 |
| HSIL | 1 | 1 | +++ | 1 |
| SCC | 1 | 1 | ++++ | 1 |
| Bacterial Vaginosis | 4 | 7 | - | 0 |
| Trichomoniasis | 3 | 3 | - | 0 |
| Atrophic /Menopause associated changes | 1 | 1 | - | 0 |
| Inadequate smear | 1 | 2 | - | 0 |
| Total | 50 | 50 | 50 | 9 |

**HISTOPATHOLOGICAL ANALYSIS**:

Out of the 50 cases studied, histopathological correlation was available only for 9(18%) cases.

The histopathological analysis of the cases reported as HSIL and SCC (in 56 and 58 year old patients respectively) by both CPS and MLBC were confirmed affirmative.

There were 5 (55.55%)NILM cases for which histopathology was done (in patients aged 38,40,40,45 and 50 years) which according to biopsy were diagnosed as Chronic Polypoidal Cervicitis. This again confirms that NILM was a correct diagnosis made by both CPS and MLBC.

Strikingly, one case(11.11%) which reported as unsatisfactory smear by CPS while it was diagnosed as NILM ,by MLBC , in a woman aged 26 years was diagnosed as Cervical Polyp by Histopathology goes in favour that all the diagnoses made by MLBC with p16 were correct as per histopathology

**STATISTICAL ANALYSIS**

The collected data was entered into MS Excel followed by analysis using SPSS version 22 (licensed to JSSAHER).

The demographic characteristics like age have been represented using arithmetic mean and Standard Deviation. The various presenting complaints were represented using percentages. The association between CPS findings and MLBC with p16 findings has been analysed using Chi Square Test.

P value less than 0.05 has been considered statistically significant.

## DISCUSSION

The objectives of the undertaken study were well fulfilled during the period of conduction of the study.

The technique used for sample collection for Conventional PAP smear commonly involves the use of wooden or Ayer's spatula. There are many other instruments that are used for cervical smear sampling like cervical brooms, endocervical brushes etc.

The technique involves taking cervical scrapings using an Ayers' spatula from the transition zone of the cervix, without making use of any lubricant while introducing the speculum into the cervix of the patient. The lubricant should be avoided in order to ensure that the lubricant does not interfere with the smear . The lubricant does not dissolve while the slide is being processed. The lubricant gets stained dark purple which might hide many cells thus contributing to an increase in the obscuring factors.

Before scraping, the wooden spatula should be dipped in saline to moisten it, this will prevent very rapid dehydration of the scraped cells.(47)The cervical sample obtained is then spread on the slide with a single lateral movement along the length of the slide.It is immediately sprayed with 95% alcohol which acts as a fixative, it is then air dried and sent to the laboratory where slide is processed. Processing of the slide involves application of the papaniculao stain, drying the slide and finally it is reported.

The procedure for manual liquid based cytology that we have followed involves the use of a cervex brush to collect material from the transformation zone or the endocervical junction by two rotations .Then the brush is detached from the handle and immersed in 5 ml of liquid fixative(5 gm of sodium chloride ,5gm of sodium citrate,50 ml of 10%formalin and 50ml of 95% alcohol) kept overnight and centrifuged at 800 rpm for 10 minutes.
The liquid fixative was prepared indigenously at the Laboratory of Department of Pathology at JSSH, Mysuru. Fixative is decanted and the excess fixative is blotted.1-2ml of polymer solution (contains agarose, polyethylene glycol, alcohol and poly-lysine) is added to tube. Vortex mixing is done, then 3-6 drops of suspension is applied to the glass slide .It is then allowed to dry and stained with conventional Pap stain. The Bethesda system for reporting cervical cytology is used.

Wooden and plastic spatulas are equally efficient in taking cervical smears. Plastic spatulas are recommended for liquid-based Pap smears as cervical cells slide off the plastic more easily than wood.(48) Cotton swabs yield very few endocervical cells as compared to the brushes.(49) Cervical broom devices are assumed to perform similarly to the combination of extended-tip spatula and endocervical brush.(48,49,50). Cervex brushes are also being used to obtain cervical smear samples, which is especially effective while using liquid based cytological analysis of the cervical samples as it improves the adequacy of the sample by collecting more amount of material for the smear owing to its increased surface area due to the bristles. This

observation of ours is in concordance with the study by Martin-Hirsch et al which states that the most commonly used Ayre's spatula is the least effective device for cervical sampling. Through their study, Martin-Hirsch et al have encouraged the use of extended-tip spatulas and cervex brushes over Ayer's spatula for primary screening and investigation of women before and after treatment for cervical intraepithelial lesions.(49)

Manual Liquid Based Cytology uses a poly l- lysine, a component of its polymer solution. We have used the manual membrane-based method; 'membrane' implies the formation of a membrane because the cells get suspended in a mono-layer. Cells are suspended in a polymer solution before it is applied to the slide. The polymer solution is composed of agarose, polyethylene glycol, alcohol and poly-L-lysine. (51) Drying of the polymer solution yields a uniform dispersion of those cells that is then sealed into a partly insoluble membrane. The membrane retains the cells on the glass slide. Soluble components are drawn out in the first phase of staining. (52) Cells get trapped in the meshwork of the polymer and are homogenously distributed within the membrane which is formed with the help of a wetting agent. The membrane gets adherent to the glass slide, not the cells.(52)

The fixative is then decanted from the centrifuged cell button.(13) In case of PAP smear, there is overlapping of cells seen and multiple layers of cells is a common occurrence. Thus the clarity of the slides is enhanced by MLBC as compared to Pap smear. An improvement in diagnostic accuracy is seen which is in concordance with the studies of Kavatkar et al.

The other components of the polymer solution used in Manual Liquid Based Cytology help in removal of various obscuring factors like blood and inflammatory cells. It helps in providing a clearer background ,reducing the chances of misinterpretation and misdiagnosis of the slides. Thereby it helps in improving the diagnostic accuracy, we did notice a better diagnosis and ease in reporting the slides which is in accordance with the literature by Maksem et al.(10)This reduces the number of visits to the hospital for the patient as the likelihood of error due to obscuring factors is minimised.

The age group that was studied majorly involved the females in reproductive and post menopausal age group. The prevalence of cervical carcinoma was seen to have increased with increase in age of the patient. This is majorly due to the age related decrease in immunity and increased susceptibility to diseases. We studied women ranging in age from 21 to 58 years. We noticed that the women in their thirties are found to have many infectious conditions like Bacterial Vaginosis and Trichomoniasis. Age of the patient and clinical history provided by her seconds the laboratory diagnosis.

Maximum complaints for which the women sought medical assistance was white discharge per vaginum (Table 4) indicating a callous attitude towards personal hygiene. The lack of personal hygiene leading to infectious conditions of the vagina in pregnant women can increase the chances of infections in the newborns, in advanced cases it might also lead to Intra Uterine Death of the baby also. Thus earlier diagnosis of these infectious conditions can aid in planning safe delivery such that the chances of transmission of infections and occurance of related morbidities can be minimised in both the mother and the child which is of paramount importance in a developing country like India . This inference which we draw from our study is in concordance with the works of Denny et al .(53)

The diagnosis of neoplastic conditions is found to be better in MLBC because of a clearer back ground, increased adequacy of sampling and lesser obscuring factors in comparison with Conventional PAP Smear technique.(10,13,49).Thus following a Screen and Treat approach towards cervical cancer prevention as proposed by Denny et al.(53)

Ancillary techniques like HPV DNA testing , Cell block and Immunohistochemistry can also be done on the remaining material after MLBC smears are made. (Figure 5)This further helps in ensuring a correct and multifaceted approach towards obtaining a correct diagnosis which also avoids the hassles of multiple sample collections for multiple techniques.This also helps in buying time which is crucial especially in cases of advanced stages like HSIL, which depending upon the nature of infecting HPV strain may or may not proceed to malignancy. HSIL is a crucial stage , which can have reactive atypia as a differential diagnosis.P16 marker is specific to those malignant cells which have been infected by the high risk strains of HPV like 16 and 18 (Figure 19).Thereby if stained by p16 immunohistomarker, the patient needs to be treated for management of the carcinoma and if negative for p16 staining , then treatment for the underlying infectious or non-malignant condition is sufficient. This saves time and is more economical for women of low and middle income countries. Especially in countries like India where cervical screening is not welcome due to fear of social stigma , minimal visits and optimal diagnosis with treatment will nip the issue at its roots.

P16 marker can however show false positives also as in cases of Trichomonas vaginalis infection, under such conditons, adequate history taking and expertise in MLBC slide analysis will help in coming to an appropriate diagnosis. We had one case of HSIL diagnosis by P16 on MLBC and it was also confirmed as HSIL by the biopsy which gives 100% accuracy which is better and comparable to the results obtained by (54) who had 92%(11 out of 12 available biopsies) of the cases reported as HSIL by p16 marker used on cell block, confirmed by biopsy. Manoli et al , referring to the article by Shidham et al have stated that P-16 $^{INK4A}$ has good specificity (SP) and positive predictive value (PPV), which also goes in favour of supporting the outcomes of our study.

On comparison of the p16 on MLBC with CPS, we found one case (2%) which showed the test method as more effective and conclusive than the conventional method. This was because, with proper technique, MLBC allows a monolayer suspension of cells with a clearer background, in which it is easier to appreciate the cellular morphological changes, which goes in accordance with the findings from the studies conducted by Jonhson et al., 2000; Maksem et al., 2001; Kavatkar et al., 2008.(6,10,12) The clear background is due removal of contaminating mucus & blood, MLBC improves the quality of screening of slides.[9,10,55,56]

The polymer solution for MLBC, used in our study was made indigenously in laboratory of Department Of Pathology , at JSSH and it contained agarose, polyethyelene glycol, alcohol and poly – L-Lysine as stated in the study by Manoli et al in 2012.[1]

Once confirmed diagnosis is obtained using point- of- care screening test like p16 marker on MLBC, both screening and treatment can be completed in a single visit. (57)Especially if the lesion is suitable for ablative treatment with cryotherapy or thermal coagulation or Loop Electrosurgical Excision Procedure(LEEP). Through our study we strongly urge the formulation

of a nationwide cervical cancer screening programme aimed at early detection and point of care treatment and screening by using one step confirmative diagnostic studies so as to prevent the problems and complications posed by lack of follow up by the patient and multiple treatment drop outs owing to lack of knowledge , low socio-economic status and fear of social stigma.

## CONCLUSION

The study revealed that manual liquid based cytology with p16 marker is a better technique in earlier diagnosing and differentiation of malignant from the pre-malignant conditions of the uterine cervix as compared to the conventional Pap Smear technique as the results obtained were statistically significant and go in order of proving the proposed hypothesis. MLBC with P16 as an ancillary technique should be employed for large scale cervical screening programmes and it can also be used as a replacement for the generally used PAP smear test.

## SUMMARY

-The study was conducted with the objective to improve the diagnosis of malignant and pre malignant conditions of the cervix.

- The two methods namely Conventional PAP Smear and Manual Liquid Based Cytology with p 16 marker were compared to analyse the ease of detection of cervical intraepithelial lesion so also to stage the carcinoma.

- The results obtained were compared with Histopathology namely Biopsy whenever possible

-The study revealed that out of the total sample size of 50, there were 10 infectious conditions, 5 normal smears, one squamous cell carcinoma, one HSIL, one koilocytic atypia, one LSIL , one atrophic smear 2 to 3 inadequate smears were also obtained and the rest of the smears were NILM.

-We reported a case in which the pap smear was unsatisfactory but the diagnosis by the p16 with NILM was in accordance with that of the histopathology findings

- We also found two cases in which, the smears prepares using the test methods were not adequate which re iterates the fact that proper training and experience in slide preparation is of paramount importance to get the correct diagnosis.

- The clear background provided by MLBC slide, makes it easier to understand the morphology of the cells better thereby ensuring a better diagnosis.

-p16 is an immunohistochemical marker which binds to the nucleus of the cells that show pre malignant and malignant transformations and stains them brown. Combining this IHC ancillary technique, we have improved the sensitivity of MLBC.

**REFERENCE**

[1] Nandini Manoli,Nandish Manoli, Lopamudra Kakoti, Akshata Kamath,Shwetha Kulkarni. Manual Liquid Based Cytology in Diagnosis of Gynaecologic Pathology.Cancer Research Journal.

[2] Zaid Alyafeai, Lahouari Ghouti. (2020) A fully-automated deep learning pipeline for cervical cancer classification. *Expert Systems with Applications* 141, 112951.

[3] Yuanhang Zhu, Chenchen Ren, Li Yang, Xiaoan Zhang, Ling Liu, Zhaoxin Wang. (2019) Performance of p16/Ki67 immunostaining, HPV E6/E7 mRNA testing, and HPV DNA assay to detect high-grade cervical dysplasia in women with ASCUS. *BMC Cancer* 19:1.

[4] Nandini M, Sherin, Devanand, Ashoka v, Nandish. Low Cost Cervical Cancer Screening Methods in Poor Resource Settings. J Gynecol Women's Health. 2017; 5(3): 555661. DOI:

10.19080/JGWH.2017.05.555661

[5] NCRP (2006). Consolidated report of population based cancer registries 2001-2004, Indian Council of Medical Research, New Delhi, India.

[6] Tewari KS, Agarwal A, Pathak A , Ramesh A , Parikh B , Singhal M, et al. Meeting report, "First Indian national conference on cervical cancer management - expert recommendations and identification of barriers to implementation". Gynecol Oncol Res & Prac 2018; 5:5. Available from:

https://doi.org/10.1186/s40661-018-0061-5.

[7] Howell LP, Davis RL, Belk TI, Agdigos R, Lowe J (1998) The autocyte preparation system for gynaecological cytology. Acta Cytol 42(1): 171177.

[8] Bishop JW, Bisner SH, Colgan TJ (1998) Multicentre masked evaluation autocyte and thinprep thin layer with matched conventional Pap, including biopsy results. Acta Cytol 42(1): 189-197.

[9] Deshou H, Changhua W, Quiyan L, et al (2009). Clinical utility of LiquiPrep cytology system for primary cervical cancer screening in a large urban hospital setting in China. J Cytology, 26, 20-5.

[10] Maksem JA, Finnomore M, Belsheim BL, et al (2001). Manual method for liquid based cytology. A demonstration using 1,000 Gynaecological cytologies collected directly to vial and prepared by a smear – slide technique. Diagn Cytopathology, 25, 334-8.

[11] Sherwani RK, Khan T, Aktar K, et al (2007). Conventional pap smear and liquid based cytology for cervical cancer screening-A comparative study. J Cytology, 24, 167-72.

[12] Johnson T, Maksem JA, Belshein BL, et al (2000). Liquid based cervical cell collection with brushes and wooden spatulas. Diagn Cytopathol, 22, 86-91.

[13] Kavatkar AN, Nagwamshi CA, Dabaks M (2008). Study of a manual method of liquid based cervical cytology. Indian J Pathology and Microbiology, 59, 190-4.

[14] Baandrup U, Bishop JW, Bonfiglio TA, et al (2000). Sampling, sampling errors and specimen preparation. Acta Cytol, 44, 944-8.

[15] Atkins KA (2003). Liquid based preparation in Gynaecological and non–gynaecological specimens, Ed Niael Kirsham, Neil A . Progress in Pathology, Chap 5; 6. Cambridge University Press, 101-14.

[16] Ozbun MA,Meyers C. Temporal usage of multiple promoters during the life cycle of human papillomavirus type 31b. *J Virol.* 1998; **72**: 2715- 2722.

[17] Doorbar J. The papillomavirus life cycle. *J Clin Virol.* 2005; **32**( suppl 1): S7- S15.

[18] Munhoz, N, Rodrigues D, Pedregosa J, Rodrigues J, Garcia Junqueira M, Yonamine, P, Pereira, S, Uezato S, Pandossio T, Martins E, Oliveira F, Cordeiro J, Bonilha J and Cury P. (2009). The Use of Molecular Markers (p16, Ki-67 and E-Cadherin) in Uterine Cervical Biopsies. The Open Pathology Journal, 3(1), pp.10-17.

[19] Halloush RA,Akpolat I,Jim Zhai Q,Schwartz MR,Mody DR. Comparison of ProEx C with p16INK4a and Ki-67 immunohistochemical staining of cell blocks prepared from residual liquid-based cervicovaginal material: a pilot study. *Cancer.* 2008; **114**: 474- 480

[20] Human papillomavirus (HPV) and cervical cancer [Internet]. [cited 2019 Oct 23]. Available from: https://www.who.int/news-room/fact-sheets/detail/human-papillomavirus-(hpv)-and-cervical-cancer

[21] Sankaranarayanan R. Screening for cancer in low- and middle-income countries. *Ann Glob Health*. 2014; **80**: 412– 417.

[22]. Ferlay J, Soerjomataram I, Ervik M, Forman D, Bray F, Dixit R, et al.GLOBOCAN 2012, Cancer Incidence and Mortality Worldwide in 2012. Lyon, France: International Agency for Research on Cancer; 2012. [Last accessed on 2015 Dec 03]. Available from: http://www.globocan.iarc.fr . [Google Scholar]

[23]. Institute for Health Metrics and Evaluation. The Challenge Ahead: Progress in Breast and Cervical Cancer. Institute of Health Metrics and Evaluation. 2011. [Last accessed on 2016 Jan21].

Available from: http://www.healthmetricsandevaluation.org/publications/policyreport/challenge-ahead-progress-and-setbacksbreastand-cervical-cancer

[24] Bobdey S, Sathwara J, Jain A, Balasubramaniam G. Burden of cervical cancer and role of screening in India. *Indian J Med Paediatr Oncol*. 2016;37(4):278–285. doi:10.4103/0971-5851.195751

[25] Cervical cancer | National Health Portal Of India [Internet]. [cited 2019 Oct 24]. Available from: https://www.nhp.gov.in/disease/reproductive-system/female-gynaecological-diseases-/cervical-cancer

[26] Cervix Cancer Facts - Rajiv Gandhi Cancer Institute & Research Centre [Internet]. [cited 2019 Oct 24]. Available from: https://www.rgcirc.org/cervix-cancer/

[27] Sreedevi A, Javed R, Dinesh A. Epidemiology of cervical cancer with special focus on India. Int J Womens Health. 2015 Apr 16;7:405–14.

[28] Welcome to National Cancer Registry Programme [Internet]. [cited 2019 Oct 24]. Available from: http://www.ncdirindia.org/ncrp/ca/chapter6_Report.aspx?SiteName=Cerv&ReportType=Int_Graph&Sex=F&MyBtn=View+Graph

[29] HTA_CaCx Screening in India.pdf [Internet]. [cited 2019 Oct 24]. Available from: https://dhr.gov.in/sites/default/files/HTA_CaCx%20Screening%20in%20India.pdf

[30] Kalof AN,Cooper K. p16INK4a immunoexpression: surrogate marker of high-risk HPV and high-grade cervical intraepithelial neoplasia. *Adv Anat Pathol.* 2006; **13**: 190-194

[31] Yu L, Wang L, Zhong J, Chen S. Diagnostic value of p16INK4A, Ki-67, and human papillomavirus L1 capsid protein immunochemical staining on cell blocks from residual liquid-based gynecologic cytology specimens. Cancer Cytopathol. 2010;118(1):47–55.

[32] Leversha MA,Fielding P,Watson S,Gosney JR,Field JK. Expression of p53, pRB, and p16 in lung tumours: a validation study on tissue microarrays. *J Pathol.* 2003; **200**: 610-619.

[33] Makitie AA,MacMillan C,Ho J, et al. Loss of p16 expression has prognostic significance in human nasopharyngeal carcinoma. *Clin Cancer Res.* 2003; **9**: 2177-2184.

[34] Klaes R,Woerner SM,Ridder R, et al. Detection of high-risk cervical intraepithelial neoplasia and cervical cancer by amplification of transcripts derived from integrated papillomavirus oncogenes. *Cancer Res.* 1999; **59**: 6132-6136.

[35] Klaes R,Benner A,Friedrich T, et al. p16INK4a immunohistochemistry improves interobserver agreement in the diagnosis of cervical intraepithelial neoplasia. *Am J Surg Pathol.* 2002; **26**: 1389-1399.

[36] Aoyama C,Liu P,Ostrzega N,Holschneider CH. Histologic and immunohistochemical characteristics of neoplastic and nonneoplastic subgroups of atypical squamous lesions of the uterine cervix. *Am J Clin Pathol.* 2005; **123**: 699-706.

[37] Mao C, Balasubramanian A, Yu M, Kiviat N, Ridder R, Reichert A, et al. Evaluation of a new p16INK4A ELISA test and a high-risk HPV DNA test for cervical cancer screening: Results from proof-of-concept study. Int J Cancer. 2007;120(11):2435–8.

[38] Kuhn L,Denny L,Pollack A,Lorincz A,Richart R,Wright T. Human papillomavirus DNA testing for cervical cancer screening in low-resource settings. *J Natl Cancer Inst* 2000; **92**: 818–25.

[39] Klaes R,vonKnbelDoeberitz M. Overexpression of p16 as a specific marker for dysplastic and neoplastic epithelial cells of the cervix uteri. *Int J Cancer* 2001; **92**: 276–84.

[40] Trunk MJ. Morphologic characteristics of p16 positive cells in cervical cytology samples. *Acta Cytol* 2004; **48**: 771–82.

[41] Negri G,Vittadello F,Romano F,Kasal A,Rivasi F,Girlando S,Mian C,Egarter-Vigl E. p16INK4a expression and progression risk of low-grade intraepithelial neoplasia of the cervix uteri. *Virchows Arch* 2004; **445**: 616–20.

[42] Hu L,Guo M,He Z,Thorton J,McDaniel LS,Hughson MD. Human papillomavirus genotyping and p16 expression in cervical intraepithelilial neoplasia of adolescents. *Mod Pathol* 2005; **18**: 267–73.

[43] Sano T,Oyama T,Kashiwabara K,Rukuda T,Nakajima T. Expression status of p16 protein is associated with HPV oncogenic potential in cervical and genital lesions. *Am J Pathol* 1998; **153**: 1741–8.

[44] Agoff NS,Lin P,Morhiara J,Mao C,Kiviat NB,Koutsky LA. P16 expression correlates with degree of cervical neoplasia: a comparison with Ki-67 expression and detection of high-risk HPV types. *Mod Pathol* 2003; **16**: 665–73.

[45] ALTS Group. A randomized trial on the management of low-grade squamous intraepithelial lesion cytology interpretations. *Am J Obstet Gynecol* 1003; **188**: 1393–1400.

[46] Wang SS,Trunk M,Schiffman M,Herrero R,Sherman ME,Burk RD,Hildesheim A,Bratti MC,Wright T,Rodriguez AC,Chen S,Reichert A, et al. Validation of p16INK4a as a marker of oncogenic human papillomavirus infection in cervical biopsies from a population-based cohort in Costa Rica. *Cancer Epidemiol Biomarkers Prev* 2004; **13**: 1355–60.

[47] Naib ZM. Pap Test. In: Walker HK, Hall WD, Hurst JW, editors. Clinical Methods: The History, Physical, and Laboratory Examinations [Internet]. 3rd ed. Boston: Butterworths; 1990 [cited 2019 Oct 29]. Available from: http://www.ncbi.nlm.nih.gov/books/NBK287/

[48]. Saslow D, Runowicz CD, Solomon D, Moscicki AB, Smith RA, Eyre HJ, et al. American Cancer Society guideline for the early detection of cervical neoplasia and cancer. *CA Cancer J Clin*. 2002;52:342–62.

[49]. Martin-Hirsch P, Lilford R, Jarvis G, Kitchener HC. Efficacy of cervical-smear collection devices: a systematic review and meta-analysis [published erratum appears in Lancet 2000;355:414]. *Lancet*. 1999;354:1763–70.

[50] Schooff M, Lawlor A. What Is the Best Collection Device for Screening Cervical Smears? Am Fam Physician. 2004 Apr 1;69(7):1661.

[51] Maksem JA, Dhanwada V, Trueblood JE, Weidmann J, Kane B, Bolick DR, *et al.* Testing automated liquid based cytology samples with a manual liquid based cytology method using residual cell suspensions from 500 Thin Prep cases. Diagn Cytopathol 2006;34:391-6.

[52] Maksem J. A manual method of liquid based cytology: Implementation, testing, cost and outcomes. American Society for Clinical Pathology Teleconference Series, 2004.

[53] Denny L, Quinn M, Sankaranarayanan R. Chapter 8: Screening for cervical cancer in developing countries. Vaccine. 2006 Aug 31;24 Suppl 3:S3/71-77.

[54] Shidham VB, Mehrotra R, Varsegi G, D'Amore KL, Hunt B, Narayan R. p16INK4a immunocytochemistry on cell blocks as an adjunct to cervical cytology: Potential reflex testing on specially prepared cell blocks from residual liquid-based cytology specimens. CytoJournal [Internet]. 2011 Jan 31 [cited 2019 Oct 29];8. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3045765/

[55] Austin RM, Ramzy I (1998). Increased detection of epithelial cell abnormalities by liquid based gynaecologic cytology preparations. A review of accumulated Data. Acta Cytol, 42, 178-84.

[56] Lee, Kelly D, Gravitt PE, et al (2006). Validation of a low cost, liquid based screening method of cervical intraepithelial neoplasia. Am J Obst & Gynaecology, 195, 965-70.

[57] Basu P, Meheus F, Chami Y, Hariprasad R, Zhao F, Sankaranarayanan R. Management algorithms for cervical cancer screening and precancer treatment for resource-limited settings. Int J Gynaecol Obstet Off Organ Int Fed Gynaecol Obstet. 2017 Jul;138 Suppl 1:26–32.