RESEARCH ARTICLE

Manual Liquid Based Cytology in Primary Screening for Cervical Cancer - a Cost Effective Preposition for Scarce Resource Settings

NM Nandini[1]*, SM Nandish[2], P Pallavi[1], SK Akshatha[1], AP Chandrashekhar[2], S Anjali[2], Murali Dhar[3]

Abstract

Conventional pap smear (CPS) examination has been the mainstay for early detection of cervical cancer. However, its widespread use has not been possible due to the inherent limitations, like presence of obscuring blood and inflammation, reducing its sensitivity considerably. Automated methods in use in developed countries may not be affordable in the developing countries due to paucity of resources. On the other hand, manual liquid based cytology (MLBC) is a technique that is cost effective and improves detection of precursor lesions and specimen adequacy. Therefore the aim of the study was to compare the utility of MLBC with that of CPS in cervical cancer screening. A prospective study of 100 cases through MLBC and CPS was conducted from October 2009 to July 2010, in a Medical College in India, by two independent pathologists and correlated with histopathology (22 cases). Morphological features as seen through MLBC and CPS were compared. Subsequently, all the cases were grouped based on cytological diagnosis according to two methods into 10 groups and a subjective comparison was made. In order to compare the validity of MLBC with CPS in case of major diagnoses, sensitivity and specificity of the two methods were estimated considering histological examination as the gold standard. Increased detection rate with MLBC was 150%. The concordance rate by LBC/histopathology v/s CPS/histopathology was also improved (86% vs 77%) The percentage agreement by the two methods was 68%. MLBC was more sensitive in diagnosis of LSIL and more specific in the diagnosis of inflammation. Thus, MLBC was found to be better than CPS in diagnosis of precursor lesions. It provided better morphology with increased detection of abnormalities and preservation of specimen for cell block and ancillary studies like immunocytochemistry and HPV detection. Therefore, it can be used as alternative strategy for cervical cancer prevention in limited resource settings

Keywords: Manual liquid based cytology - pap smear - cervical screening - low cost method

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Introduction

Cancer of the uterine cervix is the third most common cancer among women globally. Nearly half million new cases and over quarter million deaths were estimated to have occurred worldwide in 2003. About 80% of these cases and deaths occurred in developing countries (Ferley et al., 2010). Among Indian women, it is the most common form of cancer. During the year 2001-2004, proportion of cervical cancers varied from 13 to 21 percent in various urban population based registries under the network of the National Cancer Registry Programme (NCRP) of Indian Council of Medical Research (ICMR). The proportion of this cancer was 37% of all cancers among females in rural Barshi (Maharashtra) registry area. The age standardized incidence rate of this cancer in Indian population based registries varied from 13 to 25 per 100,000 women (NCRP, 2006).

Etiological associations and possible risk factors for cervical carcinoma have been studied quite thoroughly. The main risk factors reported are sexual and reproductive behaviors, socio-economic factors (like, education and income), viruses (e.g., herpes, simplex virus, human papilloma virus, HIV). Thus the accumulated evidence suggests that the cervical cancer is amenable for primary as well as secondary prevention. Sexual hygiene, use of barrier contraceptives and ritual circumcision can undoubtedly reduce cervical cancer incidence resulting in the prevention of a substantial proportion of cervical cancers. Primary prevention however depends on general awareness among women about risk factors and required changes in life style. This is a major issue in the setup of developing countries where educational level of the women is yet to improve considerably (Crum, 2007).

Therefore, the main focus is on the secondary prevention through early detection, a focus point of

[1]*Departments of Pathology,* [2]*Obstetrics and Gynaecology, JSS Medical College (A constituent college of JSS University), Mysore*
[3]*Dept of Statistics, Manipal University, Manipal, India *For correspondence: nanda_dr05@yahoo.com*

National Cancer Control Programme revised in 1985 (NCCP, 2006). Though the cytological examination has been the mainstay for early detection of cervical cancer, its widespread use has not been possible in the developing countries due to paucity of resources, man power and other facilities. Moreover, although Conventional Pap Smears (CPS) screening leads to reduction in the rate of invasive cancer of the uterine cervix, its sensitivity reduces to less than 50% when there is presence of obscuring blood, inflammation or thick areas of overlapping epithelial cells (Sherwani et al., 2007; Kavatkar et al., 2008). These problems with the CPS, gave rise to the advanced technologies, like, Thin Prep and Sure Path commonly used in the setup of developed countries, like UK and USA (Kavatkar et al., 2008; Deshou et al., 2009). Use of these technologies however are quite resource intensive and therefore not feasible in the setup of developing countries.

On the other hand, Manual Liquid Based Cytology (MLBC) is a technique that enables cells to be suspended in a monolayer and thus improves detection of precursor lesions and improvement of specimen adequacy. MLBC has been reported to 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 (Baker, 2002). Also, in case of MLBC, the residual sample can be used for other tests like detection of HPV, DNA and immunocyto chemistry thereby increasing the utility of MLBC (Sherwani et al., 2007; Kavatkar et al., 2008).

There are studies (Maksem et al., 2001; Kavatkar et al., 2008) which have dealt with liquid based cytology and have found its diagnostic accuracy comparable with conventional Pap smears. MLBC method however is specific to the laboratory, available equipments, fixatives and polymer solutions, etc.

Therefore the overall aim of our study was to assess the utility of indigenous MLBC in comparison of CPS for low resource settings. Specific objectives of the study were: a) To compare the morphological view of different diagnoses according to CPS and MLBC, b) To compare the cellular and nuclear parameters according to two methods and c) To compare the validity of the two methods in terms of sensitivity and specificity.

Materials and Methods

The study included 100 patients in the age range of 20 to 70 years whose slides were referred to Cytology Section of Dept of Pathology, JSS Hospital, Mysore, India. They were registered as OPD patients in the Department of OBG with clinical history of white discharge, and clinical diagnosis of infection or cancer. A plastic Ayre's spatula was used to collect the samples. Spatula was rotated against the ecto-cervix for a full rotation so as to include the transformation zone. Split sample method was followed wherein material from one side of the spatula was spread onto a clean glass slide and fixed by bio-spray for conventional method. The spatula was then dipped into a bottle with fixative prepared in our laboratory. The specimens were subjected to two methods for morphological diagnosis namely Conventional Pap Smear (CPS) and Manual Liquid Based Cytology (MLBC).

CPS method

This method included the standard procedure of usual staining of the glass slides with the spread smear. Rapid pap method of staining was used.

MLBC procedure

We report here an indigenous method which is specific to our laboratory using chemicals available in the laboratory, a simple equipment, fixative and polymer solution prepared by us, thus making it a low cost manual method of cervical pap smear screening. The method was accomplished in the following steps of processing.

The material collected in the liquid fixative (containing sodium chloride, sodium citrate, 10% formalin and isopropyl alcohol).was further processed after a minimum duration of 24 hours. The procedure involved first the mixing of the sample properly before transferring it to a clean test tube and centrifuging it at 1,000 rounds per minute (rpm) for 5 minutes. The supernatant was then decanted. Two millilitre of polymer solution containing agarose, polyethylene glycol, poly-l-lysine and alcohol was added to the deposit. This was further centrifuged at 2,000 rpm (600-800g) for 5-10 minutes. The supernatant was discarded and from the deposit smear was made on a clean glass slide using a Pasteur pipette. The prepared slides were fixed by drying it in an hot air oven , by keeping the slides on a metal tray for 15 minutes at 50˚C. The slides were further fixed by dipping it in 95% alcohol for 15 minutes and stained with rapid pap stain.

Observations

The smears were studied by two independent observers by using 15 parameters i.e. cellularity, background, uniform distribution, artifacts, cellular overlapping architectural and cellular morphologic change (architectural and cytoplasmic distortion, cytoplasmic vacuolation, cellular elongation, imprecise and folded cytoplasmic borders) nuclear changes (nuclear hyperchromasia, coarse chromatin, prominent nucleoli, irregular nuclear borders, atypical mitosis) and inflammatory infiltrate. The Bethesda system for reporting cervical cytology was used in both methods (Table 1).

Table 1. Morphological Features as Observed Through Conventional Pap Smear (CPS) and Manual Liquid Based Cytology (MLBC) Methods

Morphological feature	CPS	MLBC
Cellularity	Unsatisfactory in 10 cases	Adequate
Cleanliness of background	Absent	Absent
Uniformity of distribution	Present	Absent
Artifacts	Present	Rare
Cellular overlapping	Yes (Marked)	Yes (Rare)
Architectural change	Yes	Rare
Cellular morphological change	Yes	No
Nuclear change	Not always clear	Always very clear

Statistical analysis

The frequency distribution of leading morphological features was worked out to compare the same according to the two methods under study. Increased detection rate (IDR) was calculated as following, **IDR=((P_m-P_c)/P_c)*100**, where, P_m is the number of positive cases through MLBC and P_c is the same through CPS.

Subsequently, in order to compare the validity of CPS and MLBC in the diagnosis of LSIL and inflammation, sensitivity and specificity of the same were estimated considering the histopathological examination (HPE) as the gold standard method.

Results

A comparison of morphological view according to three methods for all different diagnoses (Figures 1 to 11)

Normal smears: CPS showed superficial squamous cells mixed with intermediate squamous cells of normal morphology whereas MLBC showed increase in the number of cells thus increasing the adequacy of cells for the study which was confirmed by HPE.

Inflammatory Smear: CPS showed features of inflammation with presence of blood and inflammatory cells obscuring the superficial and intermediate squamous cells whereas MLBC showed normal superficial and intermediate squamous cells with a clean background. HPE showing features of chronic cervicitis.

Bacterial vaginosis: Clue cells were seen more clearly by MLBC method.

LSIL(low grade squamous intraepithelial lesion): Inflammatory cells overlying on the superficial squamous cells obscuring the nuclear features according to CPS whereas single monolayer arrangement of squamous cells with removal of obscuring factors according to MLBC. Even nuclear enlargement was clearly seen by MLBC. This aspect with MLBC helps in detecting the precursor lesions of cervical cancer.

HSIL(high grade squamous intraepithelial lesion): CPS showed few cells covered by blood whereas MLBC showed pleomorphic cells with clear background. The diagnosis of HSIL was confirmed by HPE .

Squamous cell carcinoma: CPS showed sheets of keratinized pleomorphic squamous cells whereas MLBC showed sheets of pleomorphic squamous cells with tadpole cell in a clear background. HPE confirmed the diagnosis as a large cell keratinizing squamous cell carcinoma.

Adenocarcinoma in situ: CPS showed sheets of endocervical cells arranged in acinar pattern with palisading of cells whereas MLBC showed a clear cluster of pleomorphic endocervical cluster along with normal superficial squamous cells.

Menopausal smear: CPS showed syncytial clusters of intermediate squamous cells whereas MLBC showed a monolayer of intermediate squamous cells of normal morphology. HPE showed procidential changes.

HPV (Koilocytic atypia): Koilocytes were seen through CPS and MLBC where HPE showed koilocytic atypia at the upper layers of the ectocervix. koilocytes with intermediate squamous cells showing perinuclear halo with nuclear atypia.

Candidal infestation: CPS showed only inflammatory smear change. MLBC showed the presence of candidal pseudohyphae.

Leptotrix infection: CPS showed only normal smear whereas presence of leptotrix filamentous forms were seen more commonly by MLBC.

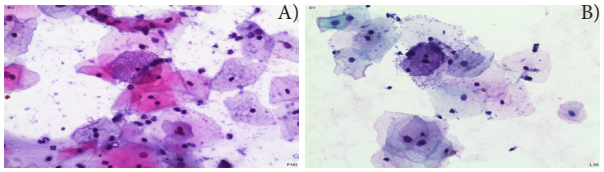

Figure 3. Clue Cell Identified by MLBC Method. A) CPS (PAP, 100X), B) LBC (PAP, 450X).

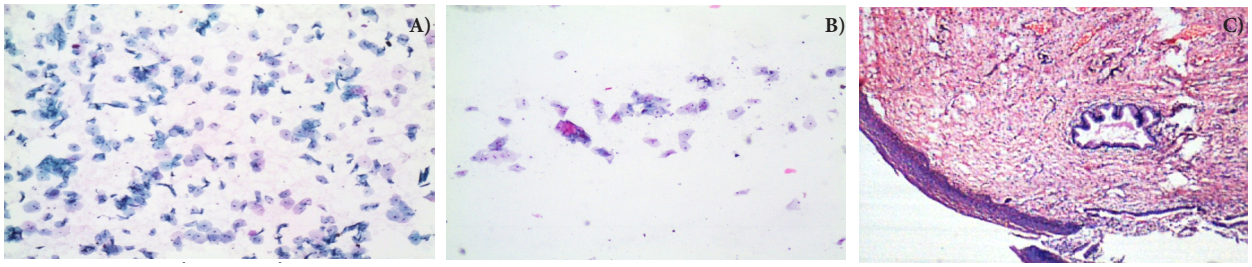

Figure 1. Normal Smear By Both CPS And MLBC Methods. Hpe showing normal cervix A) LBC (PAP, 450X), B) CPS (PAP, 100X), C) HPE (H&E, 10X).

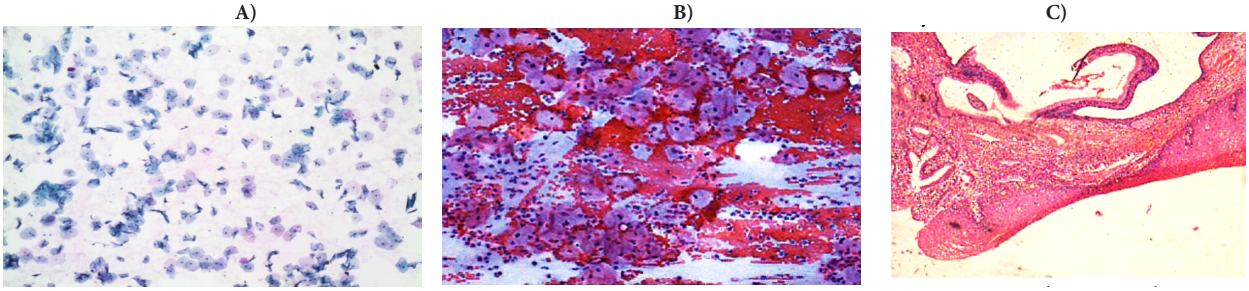

Figure 2. Inflammatory Smear by CPS, Negative for Intraepithelial Lesion by MLBC, Chronic Cervicitis by HPE. A) LBC (PAP, 450X), B) CPS (PAP, 100X), C) HPE (H&E, 10X).

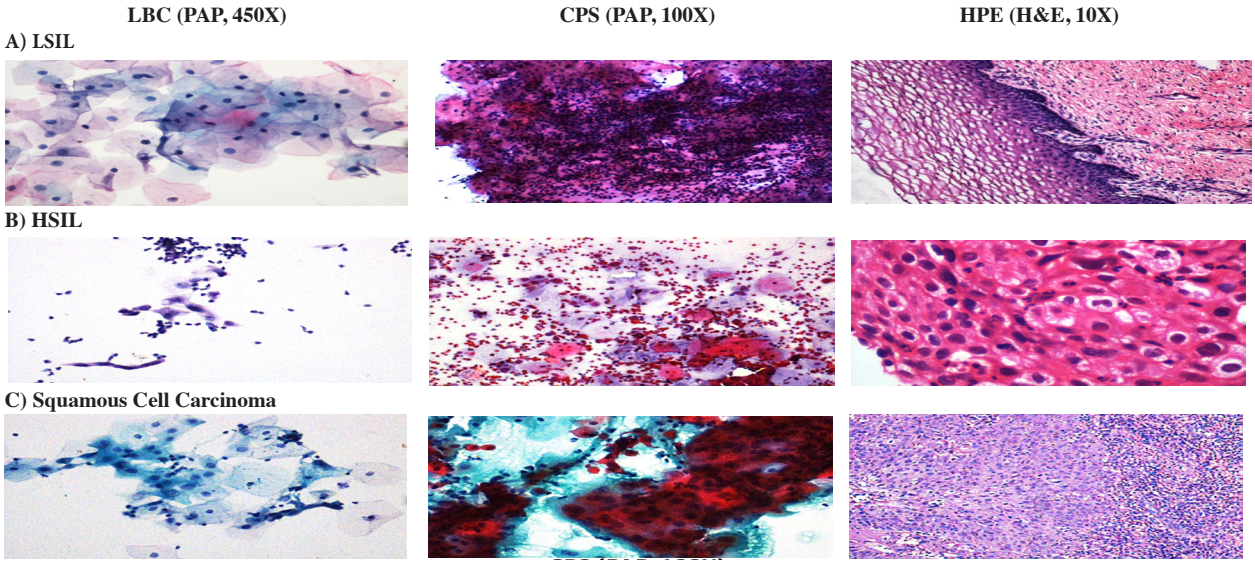

Figure 4. A) Inflammatory Smear by CPS, LSIL by MLBC, Mild Dysplasia by HPE. B) High Grade Intraepithelial Lesion Diagnosed Clearly by MLBC, Confirmed by HPE. C) Squamous Cell Carcinoma by CPS, MLBC and HPE.

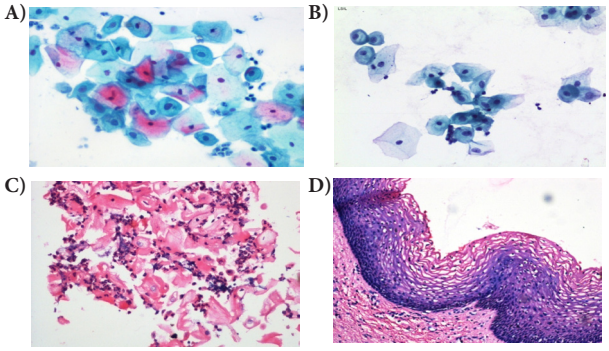

Figure 5. Koilocytic Atypia by A) CPS, B) MLBC, C) Cell Block, and D) HPE.

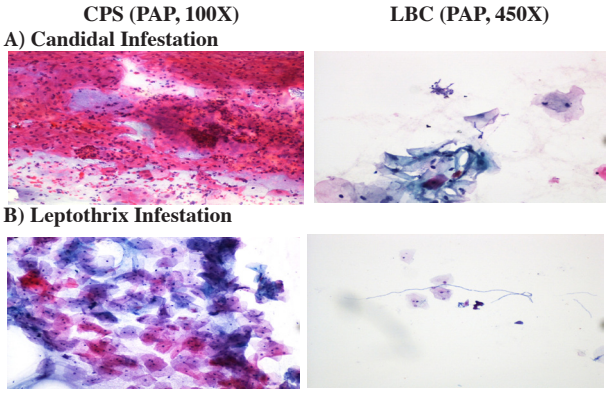

Figure 6. A) Candidal pseudohyphae seen by MLBCby HPE. B) Leptothrix seen by MLBC

Table 2. Comparison of Classification of Cases by Conventional Pap Smear (CPS) and Manual Liquid Based Cytology (MLBC) with that by Histo-Pathological Examination (HPE)

Categories	CPS	MLBC	HPE
Normal smear	14	15	1
Inflammatory Smear (NILM)	42	20	8
Bacterial Vaginosis	7	14	0
LSIL	14	36	8
HSIL	1	1	1
Squamous cell carcinoma	2	2	1
Adenocarcinoma	1	1	1
Menopausal	8	6	1
Unsatisfactory infestations	9	1	0
HPV (Koilocytic atypia)	2	3	1
Candidiasis	0	1	0
Leptothrix	0	3*	0
Total	100	100	22

*Seen in combination with NILM.

Table 3. Sensitivity and Specificity of Conventional Pap Smear and Manual Liquid Based Cytology Methods in the Diagnosis of LSIL and Inflammation

Method	Sensitivity (%)	Specificity (%)
LSIL:		
MLBC	75	100
CPS	50	100
Inflammation:		
MLBC	88.9	92.3
CPS	88.9	76.9

Comparison of cellular and nuclear parameters and the diagnoses by two methods

Panel of various cellular and nuclear parameters were compared between CPS and MLBC (Table 1). Cellularity was adequate in all of the MLBC cases whereas it was unsatisfactory in many CPS cases. The background was observed to be clean in all cases of MLBC which was not the case in majority of CPS. Uniform distribution seen in MLBC with cellular overlapping was seen more in CPS than in MLBC. Artifacts were present in most CPS samples. Architectural and cellular morphologic changes were present in most of CPS samples. Inflammatory infiltrate were prominently present in CPS but decreased in MLBC cases. Nuclear changes were very clear by MLBC, but not so clear by CPS. Diagnostic features of 100 cases according to both CPS and MLBC were divided into 10 categories (Table 2). In the study, the comparison between conventional CPS and MLBC showed certain observations in different diagnostic categories. Both the methods showed same number of normal smears. Inflammatory smears diagnosis was more by CPS than

by MLBC (42%). However, diagnosis of Bacterial Vaginosis (14%) and low grade squamous intraepithelial lesion (36%) were more by MLBC which is a significant observation. Also the number of infestations detected by MLBC method was increased ie koilocytic atypia, candiasis and leptothrix. Cytohistological correlation was done in 22 cases. Increased detection rate was 150% for low grade intraepithelial lesion.

Validity of the two methods

The rate of concordance with histology was 77% for CPS and LBC, whereas, it was 86% for MLBC. The rate of increased detection of LSIL through MLBC was 150%. In addition, to compare the validity of the two methods, we estimated sensitivity and specificity of the two considering HPE as the gold standard for the two important diagnoses, namely, LSIL and inflammation. In the diagnosis of LSIL, MLBC was more sensitive than CPS (75% vs. 50%) with similar specificity (100%). In case of inflammation also, MLBC was found to perform better being more specific (92% vs. 77%) with same sensitivity (89%).

Discussion

The conventional papanicolaou smear (CPS) has been the mainstay of screening for cervical cancer and its precursor lesions for approximately 50 years without major changes in the techniques related to preparation and interpretation. Despite its success as a preventive screening tool for cervical cancer, CPS has its limitations (Deshou et al., 2009). False negatives in CPS may be related to inadequate sampling, inadequate transfer of the sample onto the glass slide or deficiencies in the microscopic assessment of the slide (Maksem et al., 2001). To overcome these problems, a new slide preparation method namely the Manual Liquid Based Cytology (MLBC)was introduced by(.Maksem et al., 2001)

In the manual liquid based method, cells are uniformly dispersed by a membrane, from a suspension of cells in a polymer solution (Maksem et al., 2001; Kavatkar et al., 2008). As with most screening tests, the CPS suffers from imperfect sensitivity and specificity. Although a clinician may have excellent collection and sampling technique, only approximately 20% of the cells collected are smeared on the glass slide in CPS (Sherwani et al., 2007; Deshou et al., 2009; Johnson et al., 2000). Many studies have shown that with proper training, MLBC results in a higher diagnostic yield than traditional cervical smears (Baandrup et al., 2000; Atkins et al., 2003; Sherwani et al., 2007). In our study the MLBC method was found to be comparable to the conventional pap smear on some parameters and superior on few others. Liquid based cytology has recently become an alternative to CPS in the detection of intraepithelial lesions as well as in invasive carcinoma of the uterine cervix. Several reports have discussed its benefits to cytologic diagnosis.

In most of these reports a significant rise in sensitivity was achieved with liquid based procedures, without major losses in specificity (Bernstein et al., 2001; Abulafi et al., 2003; Davey & Zarbo et al., 2003; Alves et al., 2004).

The present study showed the sensitivity and specificity of CPS and MLBC for LSIL group. MLBC is more sensitive method for the diagnosis of LSIL. Similarly both the methods were sensitive to the same extent for inflammation while MLBC is more specific thus agreeing with the findings of (Berstein et al., 2001; Alves et al., 2004)

Regarding increase detection rate, our study found high increased detection rate which was consistent with earlier reports (Austin & Ramzy, 1998; Bishop et al., 1998). There was significant difference in concordance rate between CPS & MLBC Thus LBC showed a significantly higher histologically versus cytological concordance referral rate, as also observed by (Deshou et al., 2009).

Comparison of unsatisfactory smear rates have varied with some investigators reporting increased rates for liquid based methods (Maksem et al., 2001; Baker et al., 2002, Alves et al., 2004) with some investigators reporting decreased (Park et al., 2001; Baker, 2002) and some, no significant change (Park et al., 2001; Baker, 2002). In our study the number of unsatisfactory smears by CPS was 9 as compared to MLBC which had one unsatisfactory smear with scant squamous cellularity as observed in many studies

Increased detection of cellular abnormalities by liquid based method depends on many factors like adequacy of sample, the type of spatula used to collect the sample and type of sampling like whether direct to vial or split sample method.

The advantages of liquid based cytology are mainly that by the liquid based methods the specimen is collected in a preservative solution and allows long term storage of the liquid sample. The polymer solution used in our laboratory contains agarose, polyethyelene glycol, alcohol and poly – L-Lysine which help to form a cell button and form a thin monolayer of cells within a clean background (Jonhson et al., 2000; Maksem et al., 2001; Kavatkar et al., 2008). Thus by giving a clear background and removal of contaminating mucus & blood, MLBC improves the quality of screening of slides (Austin and Ramzy, 1998; Maksem et al., 2001; Lee, 2006; Deshou et al., 2009).

The sample enrichment which can be possible in a liquid preservation media helps in harvesting a more representative sample. The thin layer preparation cells functions as part of the enrichment process by reducing unreadable, thick areas where cells of interest can hide (Baandrup et al., 2000). Although LBC is recommended for cervical cancer screening, it involves the use of automated devices which are high cost per test (Maksem et al., 2001; Lee et al., 2006; Kavatkar et al., 2008; Deshou et al., 2009).

Manual method of liquid based cytology which we are following is an inexpensive, cost effective method of LBC which we have adapted and are comparing it with conventional pap smears (CPS) for its adequacy and utility The other advantages of MLBC method is that the residual specimens can be used for ancillary testing like imunocytochemistry by cell block. Preparation (Richard et al., 1999) and detection of HPV DNA by PCR or in situ DNA hybridization (Maksem et al., 2001; Lee et al., 2006; Kavatkar et al., 2008; Deshou et al., 2009).

Cell blocks from LBC specimens were found to aid in diagnosis of 20% of specimens in Thin prep preparation and were critical to the diagnosis in 5% of cases- cell blocks have the ability to reduce both the false positive and false negative rates of the lab test (Richard et al., 1999; Johnson et al., 2000).

We have 4 cases with cell block preparation where in 3 cases of Inflammatory smear and one case of Koilocyptic atypia were confirmed on cell block. Further studies are required.Immunocytochemical detection of molecular alterations caused by HPV in host cells could potentially be used as an adjunct to cytological screening to improve SE (imperfect sensitivity) without compromising SP (sufficient specificity). Considering that cytopathologists are exper morphologists and are used to interpret immunostains, an ideal tool for the detection of high-grade squamous intraepithelial lesions(HSIL) could be an immunomarker applied to a preparation derived from a liquid based sample. Immunocytochemistry is fast, simple and relatively inexpensive and provides information linked to cytomorphology. Furthermore, the evaluation of of immunocytochemistry is fast, simple, and relatively inexpensive and provides information linked to cytomorphology. Furthermore. The evaluation of immunocytochemistry applied to LBC samples could be automated (Pinto et al., 2012).

To date, a number of promising markers have been investigated. These include $p16^{ink4a}$, MIB-1,BD-ProExC, and L1. Newer possibilities involve a variety of gene products associated with aberrations of chromosome 3q, such as telomerase, p63 and PIK3CA,as well the combination of biomarkers such as $p16^{ink4a}$ and MIB-1 in the same assay.

Among the candidate immunomarkers for cervical pre-cancer lesion detection, $p16^{ink4a}$ is one of the most promising and the most studied. Several studies have tested it in either LBC or cell block preparations and majority have demonstrated the effectiveness of $p16^{ink4a}$ for improving the cytological detection of HSIL. When compared with other adjuctive tests for detection of HPV infection in a population with previous abnormal cytology, $p16^{ink4a}$ showed lower imperfect sensitivity but good specificity and the highest PPV (positive predictive value) (Szarewski et al., 2008).

Limitations of the study were that Split sample method which we followed to compare between CPS and LBC method depends on proper collection and smearing method which will enhance the effectiveness of LBC (McGoogan et al., 1998). We found that conventional cases with endocervical component were noted more than LBC which has been attributed to the split sample collection protocol & we follow the residual samples for the LBC. This can be overcome by direct sampling method (Austin et al., 1998). Advanced developed countries have automated computer assisted systems for reading of slides like the PAPNET system and Focal point analyser which increases the detection rate by reducing the screening time but is a costly method for routine screening in our country (Bergerson et al., 2000; Kavatkar et al., 2008).

In addition to increased detection of precursors of malignancies and malignancies by MLBC method, good number of infestions like Candida, Leptothrix,and HPV with koilocytic atypia can be detected as observed by many authors and us also even infections like Bacterial Vaginosis (shift to the left of vaginal flora) are detected in increases number (Papillo et al., 1998; Sherwani et al., 2007).

Certain issues will have to be dealt with to overcome the limitations of the study. Sensitivity and specificity can be better achieved when there is histopathological biopsies for the precursor lesions detected by both CPS and MLBC methods. Also introduction of cell block for the detection of precursor lesions , will help to use immunemarkers like Cytokeratin,CEA and Vimentin.so as to differentiate between endometrial carcinomas and endocervical carcinoma (Syrjanen et al., 2010). Low cost HPV detection by using liquid based cytology should be the next line of screening of cervical cancer as it has replaced conventional pap smear screening in developed countries of Europe (Syrjanen et al., 2010).

Recent concepts on screening state that since molecular HPV tests in general are more expensive and technically more challenging than immunostains. this makes it attractive to consider future screening modalities that include one or more of the biomarkers described either in combination. This concept needs further revaluation and follow up by colposcopic, histological and clinical endpoints as well as adequate follow-up (Pinto et al., 2012)

In conclusion, the low cost manual liquid based cytology method of cervical screening was found to be better than the standard commercial method. It also over comes the limitations of CPS. Thus it is of value as an alternative more effective screening strategy in low resource settings, like developing countries including India where women are at high risk for developing cervical cancer. Also, further ancillary testing like HPV and immunemarkers becomes possible in the testing of new paradigms for screening strategies that are required in such settings. Moreover, MLBC detected SIL and its precursor lesions in increased number. Hence, it can be used as alternative strategy as a measure for cervical cancer prevention in limited resource settings.

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