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Original Research Paper

Medicine

HIGH RISK HPV DETECTION IN PRECURSOR LESIONS OF CERVIX USING LBC SAMPLES.

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ABSTRACT

Background: Liquid-based cytology (LBC) was developed as an alternative to conventional cytology screening to improve specimen adequacy and sensitivity in detecting cervical abnormalities. High risk HPV (HR HPV) types 16 and 18 are responsible for about 70% of all cervical cancer cases worldwide.

Objectives: This study aimed (1) to compare the diagnostic efficiency of liquid based cytology LBC and conventional tests in detecting cervical dysplasia and (2) detection of high risk HR HPV types 16 and 18 in cervical precursor lesions using liquid based cytology samples.

Methods: A prospective comparative study was conducted from the year August 2014 – February 2019 at Basavatarakam Indoamerican Cancer Hospital and Research Institute, Hyderbad.A total of 200 women undergoing screening for cervical cancer were included in the study. HR-HPV 16 & 18 detection was done by Nested Multiplex Polymerase Chain Reaction (NMPCR).

Results: The area under the ROC curve for conventional Pap smear was 0.725 and LBC was 0.974, showing that the diagnostic accuracy of LBC method in diagnosing precursor lesions and carcinoma of cervix is better than the conventional Pap smear method. HR-HPV 16/18 were positive in 43/102 (42.2%) of cases. HPV 16 alone was positive in 32/43(74.4%), HPV 18 alone in 6/43 (13.9%) and both HPV-16 and HPV-18 were positive in 5/43 (11.6%) of the total HR-HPV positive cases.

Conclusion: The detection rate of epithelial abnormalities and infections in LBC preparation was better than CPS. LBC samples can be used for HR-HPV genotype detection to explore new paradigms of screening strategies.

KEYWORDS : Liquid based cytology, Human papillomavirus

INTRODUCTION:

Cancer of the cervix uteri is the 3rd most common cancer among women worldwide, and the 2nd leading cause of female cancer in India, with human papillomavirus (HPV) infection as well-established cause. Worldwide screening programs for cervical cancer based on the Papanicolaou (Pap) smear have contributed to the decrease in incidence and mortality of the disease [1,2,3]. Being one of the most preventable diseases, there's an estimated 96,922 new cases and about 60,078 deaths occur annually in India, representing 9.2% age-standardized mortality rate per 100,000 women [2]. This makes cervical cancer an important public health hazard.

Liquid-based cytology (LBC) was developed in the early 2000s as an alternative to conventional cytology screening to improve specimen adequacy and sensitivity in detecting cervical abnormalities, to address the limitations of Conventional Pap smear CPS and to improve its diagnostic reliability [4, 5]. High risk HPV (HR HPV) types 16 and 18 are responsible for about 70% of all cervical cancer cases worldwide [3]. Early detection of HR- HPV types and cytological screening for premalignant lesions can improve triage, treatment and follow-up in infected patients thereby preventing progression to cervical cancer [6].

This study aimed (1) to compare the diagnostic efficiency of liquid based cytology LBC and conventional tests in detecting cervical dysplasia and (2) detection of high risk HR HPV types 16 and 18 in cervical precursor lesions. There are very few studies from India using LBC as a screening test along with HR-HPV genotypes co-testing. Therefore, the present study aims to provide an insight into this much-unexplored zone.

METHODS AND MATERIALS

Study sample:

A prospective comparative study was conducted from the year August 2014 – February 2019. The study was approved by the institutional ethics committee (IEC/2019/161) at Basavatarakam Indo-American Cancer Hospital & Research Institute, Hyderabad. All the patients aged 20-70 years advised to undergo screening Pap smear, irrespective of their sexual activity and marital status, were included in the study. Women who had already received some treatment (surgery/radiotherapy) were excluded from the study.

Liquid based cytology:

A total of 200 women undergoing screening for cervical cancer were included in the study. The clinical features and findings of physical examination were noted. Cervical smears were collected by conventional method using Ayre's spatula and also by cyto brush and fixed in LBC preservative vial as per the manufacturer's instructions (U prep, Regenix, drugs Ltd.). LBC samples were then processed according to the manufacturer's guidelines.(Fig 1) The Conventional and LBC smears were stained with Papanicolaou stain [7]. The smears were then compared for cellularity, morphology, cytoplasmic and nuclear features.

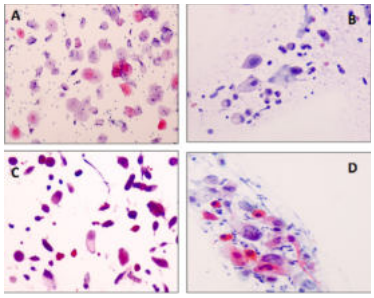

Figure 1: LBC smear showing A-NILM (100X), B- ASCUS-100X,C- ASC-H -400X, D- H-SIL-400X

HPV PCR:

The detection of HR-HPV 16 & 18 was done by Nested Multiplex Polymerase Chain Reaction (NMPCR) methodology wherein, DNA was extracted from LBC samples by Column based DNA extraction method using QIA amp DNA Mini Kit as per manufacturer's instructions and two rounds of Conventional Polymerase Chain Reaction (PCR) were carried out. Primers for PCR were selected according to the study by [8]. (Table 1)

Two rounds of PCR were performed in a final reaction volume of 20µl each. In the first round of PCR, reaction mixture contained 2µl of 10X Buffer, 2.5mM of dNTPs, 1U of DNA Polymerase (3B Blackbio Biotech India Limited from Bhopal, Madhya Pradesh, India.), 10pm each of the primers G3F, $G5BR_1$, $G6BR_2$ (Integrated DNA Technologies, Inc. US) and 200ng of DNA sample, with following PCR conditions of 94°C for 1 minute, 40°C for 1 minute, and 72°C for 2 minute for a total of 40 amplification cycles, preceded by a 4 minute initial denaturation at 94°C. The last cycle was followed by additional 10 minutes elongation step at 72°C.

NMPCR (IInd PCR) with type-specific primers (H16F, H16R, H18F and H18R of 10pM) was performed with 2ul of 1st PCR product under the following conditions: 35 cycles of 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 45 seconds. The first cycle was preceded by a 4 minutes denaturation step and the last cycle was followed by a 4 minutes elongation step. PCR products were separated by 2% agarose gel electrophoresis and the band sizes were confirmed by running DNA ladder and positive controls (Siha- HPV 16, Hela- HPV-18) (Fig 2). Based on amplicon sizes, HR-HPV 16 & 18 (table 1) were confirmed by DNA sequencing.

Table 1 : Sequences of specific forward and reverse primers used in the study

S. no	Code	Oligo seq 5'-3'	Expected size of amplicons
1	GP-E6-3F	GGGWGKKACTGAAATCGGT	630 bp
2	GP-E7-5B	CTGAGCTGTCARNTAATTGCTCA	
3	GP-E7-6B	TCCTCTGAGTYGYCTAATTGCTC	
		(W- A/T ; Y- C/T; K-G/T; N-A/C/G/T;R-A/G)	
4	H16F	CACAGTTATGCACAGAGCTGC	457 bp
5	H16R	CATATATTCATGCAATGTAGGTGTA	
6	H18F	CACTTCACTGCAAGACATAGA	322 bp
7	H18R	GTTGTGAAATCGTCGTTTTTC	

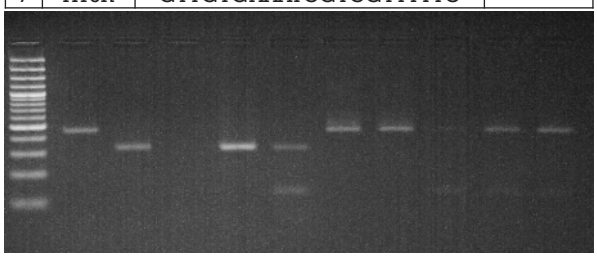

Figure 2: Lane M – DNA ladder, 1-SiHa, 2-HeLa, 3-No template control, 4 to 10-Samples

Samples in wells 4 and 5 are positive for HPV 18 as the band size is matching with corresponding band in positive control sample HeLa for HPV-18. Similarly samples in wells 6, 7, 9 and 10 are positive for HPV-16 as the band size is matching with that of the positive control SiHa for HPV-16. The sample in well 8 is showing minimal non specific amplification and is negative for HPV-16 and HPV-18.

Statistical Analysis:

Sensitivity, specificity, positive predictive value and negative predictive value were calculated. Data analysis was done using SPSS statistical software version 20.0. Pearson Chi-square test was used to analyze the data and p value was calculated wherever required. Receiver operator characteristics curve (ROC curve) was plotted to compare the diagnostic accuracy.

RESULTS:

Age wise distribution of patients:

In the present study 200 women aged 20 to 70 years who underwent screening Pap smear were evaluated. The mean age was 47.6 years. Majority of women were in the third or fourth decade of life with 65 (32.5%) belonging to the age group 46-55 years and 64 (32%) in the age group 36-45 years. Seven (3.5%) women were more than 66 years of age who were included in the study

Distribution of patients with respect to the presenting complaints:

Seventy five percent (150/200) of women included in the study for screening were with no presenting complaints. Bleeding per vaginum (PV) and postmenopausal bleeding were noted in 8.5% (17/200) and 8% (16/200) of the patients respectively. Discharge PV, irregular bleeding PV and post coital bleeding were observed in 4% (8/200), 3% (6/200) and 1.5% (3/200) of the patients respectively. It was observed that the average time needed to screen a LBC slide is 2.5-3 minutes as compared to CPS, which is at least 5-6 minutes.

Comparison of cytology results obtained by CPS and LBC:

There was a significant decrease in the number of unsatisfactory cases samples from 14 (7%) in CPS as compared to only 2 (1%) in LBC. The main cause of unsatisfactory smears was excess blood in 10/14 (71.4%) in CPS and no such samples in LBC samples. The second major cause was low cellularity in 4/14 (28.6%) cases in CPS and 2/2(100%) in LBC samples.

NILMs were more frequently diagnosed in LBC with 123(61.5%) cases as opposed to 93(46.5%) in LBC. The number of cases with atypical squamous cells (including ASCUS and ASC-H) showed a significant difference with 43(21.5%) cases in CPS and 29(14.5%) in LBC. This can be attributed to the better preservation of cell morphology in LBC. Three (1.5%) and 9 (4.5%) cases of HSIL were diagnosed with CPS and LBC respectively. Squamous cell carcinoma (SCC) and highly suspicious of SCC were seen in 24(12%) cases with CPS as compared to 20(10%) cases with LBC. AGC was reported in 23(11.5%) cases with CPS and 17(8.5%) cases with LBC. It was observed that further categorization into AGC favors neoplastic was better with LBC with 9(4.5%) cases placed in this category as opposed to 6(3%) cases with CPS (Table 2). The difference between the two techniques for diagnosis of different Bethesda categories is significant statistically (p= 0.0015).

Table 2: Comparison Of Conventional Pap Smear And Liquid Based Cytology Results

Diagnosis	CP	LBC	
	No. (%)	No. (%)	p-value
Unsatisfactory	14 (7)	2 (1)	0.0015
NILM	93 (46.5)	123 (61.5)	
ASCUS	30 (15)	19 (9.5)	
ASC-H	13 (6.5)	10 (5)	
HSIL	3 (1.5)	9 (4.5)	
SCC	24 (12)	20 (10)	
AGC	17 (8.5)	8 (4)	
AGCfavour neoplastic	6 (3)	9 (4.5)	
Total	200	200	

Comparison of benign cytology results with CPS and LBC:

Smears with altered flora and bacterial vaginosis (2/200 cases) and mycotic inflammation suggestive of *Candida*

species (6/200 cases) were seen equally in CPS and LBC. Candida hyphae were very conspicuously seen in LBC samples as compared to CPS with "Shish-kebab" like appearance. Atrophic smears were 10/200 cases in CPS and 9/200 cases in LBC. NILM cases were more frequently diagnosed with LBC (123/200) as compared to CPS (93/200) (Table 3). By using Chi-square test, p-value = 0.005, this difference was statistically significant.

Table 3: Benign Cytology Results Obtained By Cps And Lbc

Diagnosis	CPS	LBC	p-value
Mycotic inflammation	6	6	0.005
Bacterial vaginosis	2	2	
Atrophic smear	10	9	
Inflammatory smear	21	8	
NILM	54	98	
Total	93	123	

Comparison of diagnostic accuracy of CPS and LBC:

Sensitivity and specificity of conventional pap smears was 77.8% and 79.3% and for LBC was 81.8% and 95.6% respectively. The area under the ROC curve for conventional Pap smear was 0.725 and LBC was 0.974,showing that the diagnostic accuracy of LBC method in diagnosing precursor lesions and carcinoma of cervix is better than the conventional Pap smear method.

HPV RESULTS:

A total number of 93 precursors and 9 highly suspicious of SCC along with 11clinically suspicious cytologically normal smears were processed for HPV detection. Of these 8/9 (88.9%) cases of SCC, 15/60 (25%) cases of NILM, 10/17 (58.8%) cases of ASCUS, 4/5(80%) cases of ASC-H, 3/5 (60%) cases of AGC, 1/3 (33.3%) case of LSIL and 2/3(66.6%) cases of HSIL were either positive for HR- HPV 16 &/ 18. However, 45/60 (75%) of NILM, 7/17(41.2%) of ASCUS, 1/5(20%) of ASC-H, 2/5 (40%) of AGC, 2/3 (66.7%) of LSIL, 1/3 (33.3%) of HSIL, and 1/9 (11.1%) of highly suspicious of SCC were negative for both HPV-16 and HPV-18 (table 4).

Table 4: Hpv Detection In Precursor Lesions And Highly Suspicious Of Squamous Cell Carcinoma

Diagnosis	No. of cases (n=102)	HPV-16 &/18 Positive	Negative for HPV-16 & HPV-18	p-value
NILM	60	15 (25.0%)	45 (75.0%)	0.001
ASCUS	17	10 (58.8%)	7 (41.2%)	
ASC-H	5	4 (80.0%)	1(20.0%)	
AGC	5	3 (60.0%)	2 (40.0%)	
LSIL	3	1 (33.3%)	2 (66.7%)	
HSIL	3	2 (66.7%)	1(33.3%)	
Squamous cell carcinoma	9	8 (88.9%)	1 (11.1%)	

Bethesda categories and HPV subtypes:

HPV 16 alone was positive in 32/43(74.4%), HPV 18 alone in 6/43 (13.9%) and both HPV-16 and HPV-18 were positive in 5/43 (11.6%) of the total HR-HPV positive cases (Table 5). Of the 15 NILMs positive for HR-HPV, 4 were clinically suspicious, remaining 11 were found to be NILM on a repeat Pap smear after an interval of 3 months. Five of ASCUS, 4 of ASC-H, 2 of HSIL cases positive for HR-HPV were proven to be CIN3 or higher lesions on subsequent biopsy specimen. One LSIL positive for both HR-HPV types was clinically suspicious. No histopathological follow-up data was available for the remaining cases.

Out of 32/43 HPV-16 positive cases, 13 cases were proven to be SCC on subsequent histology follow-up including 1 case of extensive squamous carcinoma in situ with small foci of invasion and 2 clinically suspicious cases. No histopat hological follow-up was available for the remaining 17 cases. Out of 6/43 HPV-18 positive cases,1 (16.7%) were found to be moderately differentiated adenocarcinoma and 1 (16.7%) as poorly differentiated SCC on subsequent cervical biopsy. No statistical significant association was observed with HR-HPV 16 or 18 subtypes with different Bethesda categories, probably due to the limited number of samples subjected to HPV genotype testing (Table 5).

Table 5: Type Of Hr- Hpv Detected In The Lbc Samples

Diagnosis	No. of cases	HPV-16 Positive	HPV-18 Positive	HPV-16 & HPV-18 Positive	p-value
NILM	15	10 (66.7%)	2 (13.3%)	3(20.0%)	0.398
ASCUS	10	8 (80.0%)	1 (10.0%)	1(10.0%)	
ASC-H	4	3 (75.0%)	1 (25.0%)	-	
HSIL	2	2 (100%)	-	-	
LSIL	1	-	-	1(100%)	
AGC	3	2 (75.0%)	1 (25.0%)	-	
Squamous cell carcinoma	8	7 (87.5%)	1 (12.5%)	-	
TOTAL	43	32	6	5	

DISCUSSION:

Cervical cancer is the 2nd most common female cancer and the 2nd leading cause of cancer deaths in women aged 15 to 44 years in India [2]. In the present study age range of patients was 20- 70 years, with majority of women in the third (32.5%) and fourth (32%) decade of life in concordance with other studies [9]. The rate of unsatisfactory smears was reduced from 7 % (14 cases) in CPS to 1% (2 cases) in LBC, consistent with other studies [9,10,11,12] where the unsatisfactory rate was reduced from 0.33 to 0.18, 4.3% to 1.7% , 7.5% to 3% and 7.1% to 1.6%. In contrast [13] have reported no difference of unsatisfactory smears between CP (8%) and LBC (7%). In the present study, the most common reason for unsatisfactory smears in CPS was excessive hemorrhage followed by low cellularity and other obscuring factors like polymorphs/ inflammation and mucus. There was no inadequate LBC sample due to excess blood or other artifacts or technical errors. LBC is preferred for samples with excess blood. According to the study conducted by Chinaka et al 2014 [14] on 300 samples, the most common cause of unsatisfactory smear on LBC was scanty cellularity and on conventional Pap smear, thick smear was the commonest cause.

There was significant statistical significance (p=0.0015) between the two techniques in detection of cells from normal to carcinoma (Table 2). This can be attributed to the better preservation of cell morphology in LBC thereby providing a more accurate diagnosis and removing the uncertainty of undetermined significance of atypical cells and the diagnostic dilemma between the Bethesda categories of ASCUS, ASC-H and HSIL. Twelve cases (12/30) cases of ASCUS in CPS were associated with severe inflammation and were confirmed to be NILM on LBC. The atypical appearance of cells in CPS could be due to reactive atypia and obscuring of minute cellular details by dense inflammation, which was removed in LBC leading to a better view of cellular morphology. Three cases (1.5%) with CPS and 9 (4.5%) in LBC were HSIL, showing 3-fold increase in diagnosis of HSIL with LBC. Six cases of ASC-H on CPS were confirmed to be HSIL on LBC thereby proving that LBC samples aid in a better clarity of diagnosis by removing the uncertainty. SCC was seen in 24(12%) cases with CPS as compared to 20(10%) cases with LBC. This difference was seen due to the absence of the classical tumor diathesis with dirty, necrotic and hemorrhagic background in LBC samples. AGC was reported in 23(11.5%) cases with CPS and 17(8.5%) cases with LBC. It was observed that further categorization into AGC favoring neoplastic nature, was better with LBC with 9(4.5%) cases placed in this category as opposed to 6(3%) cases with CPS. The findings in other studies were variable depending on the number of study

subjects included, the type of study and the technique used. [15,14, 10, 16].

Sensitivity and specificity of CPS and LBC are comparable with other studies [17,18,19,14,20, 15,21]. The present study had PPV of 90 % for LBC method, which was comparable to PPV of 97.5% as observed in the study by Karimi et al 2013 [20]. The NPV of LBC method was 91.6% in the present study, which was similar to the study by Macharia et al 2014 [21] (90%). Therefore, the accuracy of LBC method in diagnosing precursor lesions and carcinoma of cervix is better than the CPS conventional Pap smear.

Various studies have shown that LBC liquid-based cytology is more effective in the detection of cervical intraepithelial neoplasia (CIN), reduces the number of unsatisfactory specimens, and reduces screening time compared to conventional cytology [15,4,5,22,23]. As observed in this study, the average time for LBC slide was 2.5-3 mins and CP slide was 5-6 mins. The observations were similar to those by Sharma et al 2016 [13] who reported screening time for CPS was 4.0 ± 0.65 min while it was 2.0 ± 0.08 min for LBC.

HR-HPV was positive in 42.2% (43/102) and negative in 57.8% (59/102) of cases. Epithelial abnormalities under Bethesda categories showed significant statistical ($p=0.001$) association with HR-HPV (Table 4). In the present study, amongst the cases reported as ASCUS on cytology, 10/17 (58.8%) were HR-HPV positive. Castle et al 2005 [24] reported 48.0% of women with ASCUS cytology were PCR positive for oncogenic HPV and HPV16 was the most common genotype among women with ASCUS (14.9%). Pankaj et al 2018 [12] reported 28.57% were HPV positive with ASCUS cytology. Evans et al 2006 [25] reported 53% of samples as HR-HPV positive diagnosed as ASC-US and Fujiwara et al 2019 [26], reported 289 (53.3%) cases, ASC-US with HPV positivity. All 5 ASC-H cases were HR-HPV positive, on subtyping, 3 (60.0%) were positive for HPV 16 and 1(20%) for HPV 18, similar to Evans et al 2006 [25]. The single ASC-H case that was negative for HPV16/18 was positive for HPV 35, another oncogenic HPV type, by Sanger's sequencing (done by Genelab) thereby confirming that absence of HR-HPV 16 or 18 does not rule out the infection by other high-risk oncogenic subtypes of HPV. All 4 cases of ASC-H that were positive for HR-HPV were proven to be CIN 3 or higher lesions on subsequent biopsy Therefore, combining HR-HPV genotype testing with LBC screening in Bethesda categories of ASCUS and ASC-H would aid in the early detection management of such lesions. The ASCUS-LSIL Triage Study (ALTS) Group concluded that after diagnosis of ASCUS, a single HPV test (on the existing sample) could identify 92.4% of CIN3. The study also shows that testing for HPV in women with ASCUS has a negative predictive value of 99.5%, reassuring that a significant abnormality is not present, and these women may be spared unnecessary colposcopy and follow-up [27]. ALTS found HPV testing to be ineffective for triage of women with LSIL as the HPV positivity rate was in excess of 80% leading to congestion at colposcopy. The authors concluded that LSIL was best managed by direct referral [28]. HR HPV positive HSIL cases were 66.6% in this study as compared to 77% reported by Barodawala et al [9] and 66.7% by Prakash P et al 2014 [29]. Only 3 cases of HSIL were found in this study so HR-HPV positivity is less compared to other studies [25,26].

HPV detection in NILMs was reported to be 10% and 27% by Banna et al 2014 [30] and Evans et al 2006 [25] respectively, similar to the present study as 25%. Prakash P et al 2014 [29] reported 20.8% HR-HPV positive in normal cytology by type specific HPV PCR method. HPV positivity of 5.37% was reported by Pankaj et al 2018 [12] but no details were furnished regarding testing methodology and in which referral lab was it done. According to the ICO HPV Information Centre (update till June 2014), HPV prevalence in normal cytology is 5%. The studies included in this meta-analysis (done by ICO HPV information centre), were of larger sample size compared to this study. Large studies with type specific PCR for HPV detection had higher prevalence of HPV in normal cytology [2]. In normal cytology 66.7% of high risk hpv positive was observed similar to Barodawala SM et al 2019 [9] of 60.7% being high risk. This high incidence of HR-HPV infection in the asymptomatic normal population of women with normal cytology indicates the hidden iceberg of HPV infection which if picked up early could prevent progression of the disease by regular follow up wherever required and adequate management. Similarly said by Sankaran arayanan R et al 2009 [31] that a single round of HPV testing was associated with a significant reduction in the number of deaths from cervical cancer.

The present study compared the performance of LBC and Conventional Pap smear in Indian scenario. The detection rate of epithelial abnormalities and infections in LBC preparation was better than CPS and this difference was statistically significant. The rate of unsatisfactory smears was significantly decreased in LBC as opposed to CPS method. Liquid based cytology was found to have high diagnostic accuracy compared to conventional cytology in the present study. HR-HPV 16 and 18 were detected in precursor lesions, highly suspicious and suggestive of carcinoma lesions and also in normal smears. Because of the small sample size in this study, the results of statistical analysis were limited. LBC samples can be used for HR-HPV genotype detection to explore new paradigms of screening strategies in the Indian scenario where women are at high risk for developing cervical cancer. However, the cost-effectiveness of LBC as compared to CPS needs to be reconsidered, especially in the absence of HPV testing as a co-screening test in a majority of centers. For women who are screened less frequently than recommended, a combination of LBC method of Pap smear coupled with HR-HPV testing will contribute to a more efficient control of screening for cervical cancer. Different Testing methods account for variability of HPV prevalence, which can be reduced by a larger study population with inclusion of all high risk HPV genotyping and adoption of a type specific sensitive method in low cost resource setting thereby, improving the screening compliance rates.

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