Detection of Human Papilloma Virus DNA from Dry Paper Cervical Smear- a Hospital Based Study

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***Abstract*- Background: Persistent infection of uterine cervix by human papilloma virus (HPV) among which high risk HPV (16, 18, 31, 33, 35, 52, and 58 collectively) are associated with cervical carcinogenesis. Cervical cancer precedes a precursor stage termed as cervical dysplasia or intraepithelial neoplasia which takes long years for development of invasive cancer.**

Aim: To studythe prevalence of HPV infection in uterine cervix and to correlate the HPV status of the cervical scrapping with the routine method of cervical cytology by Papanicolaou stain.

Methodology: The studyincluded 226 no ofcervical scrapping from married non pregnant women attending gynecology OPD for both Pap smear and PCR testing to detect HPV DNA. The Nested PCR using primers for L1 consensus gene with My09/My11 & GP6+/GP5+ used for screening HPV followed by multiplex PCR which was carried out to detect HPV 16 & HPV18 by respective primers.

Result: One hundred and ninety nine Papsmears were reported as NILM and 27 smears showed presence of SIL and invasive cervical carcinoma. PCR results detected9.7% of the patientsto be HPV positive out of which 5.3% cases were HR HPV 16 positive, 2.6 % cases were HPV 18 positive and 1.8% cases were HPV positive other than HPV 16 & 18.The sensitivity and specificity of the study were0.8182 (95%CI 0.5974-0.9481) and 0.9559 (95% CI 0.9178-0.9796) with positive predictive value being 0.6667(95% CI 0.4600-0.8349).

Keywords- Human Papiloma Virus; Pap Smear; Nested PCR; Dysplasia; SIL

I. INTRODUCTION

Human papilloma virus (HPVs)es are a family of viruses that are extremely common worldwide. They are deoxyribonucleic acid (DNA) viruses that infect skin or mucosal cells. There are more than 100 types of HPV. At least 13 of these types are oncogenic (cancer-causing) [1]. . HPV is estimated to cause 100% of cervical cancer cases, 90% of anal cancer cases, 40% of cases of cancers of the external genitalia (vulva, vagina and penis), at least 12% of oropharyngeal cancer cases, and at least 3% of oral cancer cases. HPV types 16 and 18 cause approximately 70% of all cervical cancers worldwide [1]. Carcinoma of cervix is the 2nd most common cancer among women worldwide with an estimated 5, 24000 new cases. Every year, it accounts for 80% of cases in developing countries [2]. In U.S.A. both the incidence and mortality rate has declined significantly, and largely attributable to the introduction of cervical cytology screening since 1940[3]. Cervical cancer, once a leading cause of cancer related death in women in U.S. now ranked 13th. Even now, an estimated 11,150 women are still diagnosed each year with invasive cervical cancer and approximately 3,670 die of from the disease [4]. Cervical cancer ranks 1st in many developing countries lacking cervical cytology screening programme. Cervical cancer is the second most common cause ofdeath among women in the world and the first in most developing countries [5]. The global incidence of cervical cancer has been estimated to be in the range of 493,000 to 510,000 newly diagnosed cases annually [4]. Approximately 83% of cervical cancers occur in resource–constrained populations [4]. A wealth of epidemiologic, clinico pathologic and molecular biologic data hasnow established a strong etiologic relationship between HPV infection and cervical carcinoma. Walboomers *et al*. (1999) established the presence of HPV in virtually all cervical cancers they studied using PCR based test [6].

Cervical cancer is the leading cancer among women in terms of incidence rates in 2 out of the 12 population based cancer registries (PBCRs) in India, and has the second highest incidence rate after breast cancer in the rest of the PBCRs [7]. The age-adjusted incidence rate (AAR) is highest in Chennai, a metropolitan city in south India, and lowest in Thiruvanathapuram, the capital of Kerala [7]. Carcinoma of the uterine cervix is the second most common cancer in the districts of Dibrugarh, Kamrup Urban and third in Silchartown with AAR of 11.8%, 13.1% and 13.9% respectively (ICMR, 2003-2004) of Assam [8]. Despite the high incidence of cervical cancer reported from Assam, a state in north east India, population based or hospital based studies on the HPV prevalence and genotypic distribution are very few from this region. A PCR based study detected 24.0% HPV prevalence among suspected cervical cancer patients attending Silchar Medical College at Silchar, Southern Assam (Ghosh. S. K *et al*., 2011) [9].

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Hence the aimof the study was to detect HPV infection by PCR and to correlate the HPV status of the cervical scrapping collected in Whatman 3mm filter paper with the routine method of cervical cytology by Papanicolaoustain .

II. MATERIALS & METHOD

The patient population comprised of 226 cervical scrapping collected from women aged 20-55 years attending GOPDs of Gauhati Medical College Hospital. The study was approved by Institutional Ethical Committee of GMCH vide letter No.MC/190/2007/Pt-1/71 dated 15/12/10.

A. Specimen Collection for Cervical Cytology by Pap Stain

Cytological samples have been collected using cotton tipped applicator stick/Ayer's spatula after visualizing the cervix using the Cusco's speculum from both ecto&endo cervix including transformation zone onto clean grease free labeled glass slides, fixed immediately in absolute alcohol. The smears werestained using Papanicolaou stain and examined by light microscope. The smears were reported according to the Bethesda system, which classifies cervical smears as NILM (no intra epithelial lesion/malignancy and SIL (squamous intra epithelial lesion). SIL is again graded according to the severity as low grade (LSIL) or high grade (HSIL). Cytological evidence of invasion is termed as invasive cervical cancer (ICC) [10].

B. Specimen Collection for HPV DNA in dry filter paper

Cervical scraping from the posterior vaginal pool smeared and dried on to Whatman 3MM filter paper, and stored individually at room temperature in air-locked zip-lock bags after labeling for HPV DNA testing by PCR. The dry paper smears were carried to Regional Medical Research Centre (RMRC) for North East India situated at Dibrugarh, for detection of HPV DNA by PCR. The HPV DNA PCR was carried out according to the method described by Kailash*et al.,* (2002) [11]andSotlar K *et al,*.(2004) [12].

C. Methodology for HPV DNA Testing by PCR

1) DNA Preparation:

A small piece (2-3 mm) of the dried paper specimens was punched out with the help of a sterile puncher, transferred into 1.5 ml micro centrifuge tube (eppendorf, Hamburg, Germany) containing 100 µl of distilled water. This way all the samples were made ready for DNA extraction. Then the centrifuge tubes containing the punch of the dry smears were boiled for 15 minutes. The tubes wereimmediately placed on ice for 5 minutes followed by centrifuge at 10,000 rpm for 3 minutes at 4^0C. About 70 µl volume of supernatant was transferred into a fresh 1.5 ml centrifuge tube and kept at -20^0C for PCR.

2) PCR Amplification and Amplicon Detection:

Nested-PCR for HPV detection was carried out using primers for consensus L1 gene of HPV. First round of PCR was carried out using primers MY09/11 (Baay et al.1996) [13] and second round PCR was performed using GP5+/6+ (Evans et al. 2005)[14]..First round PCR was performed at a 20 µl reaction volume using 2x PCR master mix containingTaq DNA Polymerase (Promega, USA), forward and reverse primers (MY09/MY11) at 0.5 µM final concentration, 2 µl sample and nuclease free water was added to make a final reaction volume of 20 µl. Amplification of DNA by conventional PCR was performed in a thermal cycler (Veriti 9902, Applied Biosystem, Singapore) PCR. Amplification was performed with the following cycling profile: Initial denaturation at 95^0C for 5 minutes followed by 40 cycles of 1-min denaturation at 94 ℃, 1-min annealing at 55 ℃, and 1-min elongation at 72°C. The last cycle was followed by a final extension step of 7 minutes at 72^0C. HPV 16 plasmid DNA served as positive controls, whereas nuclease free water served as negative control.The expected amplicon size of first round PCR was 450 bp. Second round PCR was performed with 1 µl of first round amplicon using primers GP5+/6+ under similar PCR conditions as the first round. The expected amplicon size of the second round PCR was 150 bp.After PCR, 10 µl of the amplified products were run on an ethidium bromide-stained 2% agarose gel (BioRad, USA) and visualized with a UV transilluminator .The gel picture was photographed in a gel documentation system (GELLOGIC 2200 Image System, Kodak, USA).Strict laboratory precautions and control measures were followed to avoid cross-contamination and carry-over in the PCR assay.

TABLE I RESULTS OF HPV STATUS OF THE PATIENT WITH EPITHELIAL CELLABNORMALITY

Category / HPV Status	**NLIM (4/199) 2.01%**	**ASCUS (0/3) 0%**	**LSIL (4/8) 50.0%**	**HSIL (8/8) 100%**	**SCC (6/8) 75.0%**	**Total (22/226) 9.7%**
HPV 16	1	0	1	4	6	12 (5.3%)
HPV 18	1	0	1	4	0	6(2.6%)
HPV Other than 16/18	2	0	2	0	0	4(1.8%)

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3) Sub Typing of HPV:

HPV sub typing was performed on positive samples using another nested PCR for the subtypes HPV 16 and HPV 18. Here first round PCR was done using primers GP E6 3F (common primer) and GP E6 5B (for HPV 16) in one set and GP E6 3F (common primer) and GP E6 6B (for HPV 18) in another set applying same PCR protocol and profile in both sets [12].. The 2nd round of nested PCR was processed using amplicons of the first round as template in both the sets using HPV16 F &HPV16 R and HPV18 F & HPV18 R respectively. Amplifications were performed with the following cycling profile: Initial denaturation was performed by incubation at 95^0C for 5 minutes followed by 40 cycles of 30 seconds denaturation at 95 ℃, 40 seconds annealing at 50 ℃, and 40 seconds elongation at 72 ℃. The last cycle was followed by a final extension step of 5 minutes at 72^0C. The sizes of amplified PCR products were observed against a 100bp DNA ladder. The expected product size for HPV16was 457bp and 322bp for HPV 18 (Cite Figure 1 & 2).

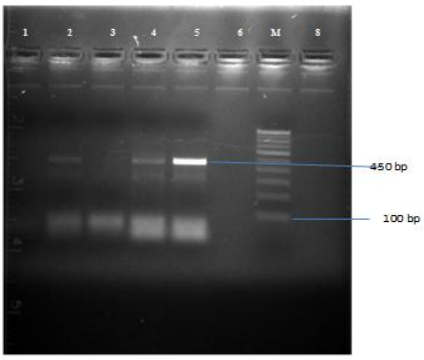

Fig.1: Post PCR agarose gel electrophoresis showing presence of HPV in lane 2 and 4. Lane 5 = PC, lane 6= NC and M =100 bp DNA

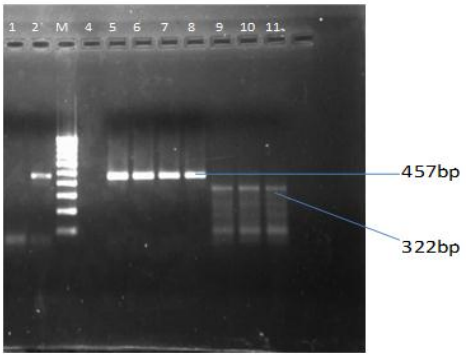

Fig.2: Type specific PCR for HPV16 (457bp at Lane5,6,7&8); Lane1=NC for HPV18, Lane 2=PC for HPV16, Lane4=NC for HPV16;Lane 9, 10 & 11 showing presence of HPV18(322bp)

The sequences of the primers used for the present study are described in Table2.

TABLE II THE SEQUENCES OF THE PRIMERS

HPV genotype	Amplicon (bp)	Sequence (5`-3`)
MY09/11	450	FP- GCM CAG GGW CAT AAY AAT GG
		RP- CAA CTT CAT CCA CGT TAC ACC
GP 5+/6+	150	FP- AAT GCC TGT GTT CAT TGC TG
		RP- TTC AAG GTC AGC CCC TAC AC
16	457	CAC AGT TAT GCA CAG AGC TGC
		CAT ATA TTC ATG CAA TGT AGG TGT A
18	322	CAC TTC ACT GCA AGA CAT AGA
		GTT GTG AAA TCG TCG TTT TTC A

GP-E6-6B	HPV genotype	GP-E6-3F	GP-E6-5B
Consensus	GGG WGK KAC TGA AAT CGG T	CTG AGC TGT CAR NTA ATT GCT CA	TCC TCT GAG TYG YCT AAT TGC TC
Common	—— C— A— —— —T —— –		
16		—— —— —— —T T— —— —— —	
18			— —— —— —— –T– —— —— —

Single-letter code: W, A/T; K, G/T; R, A/G; Y, C/T; N, A/C/G/T; X, unknown nucleotides, –identity with consensus sequence.

4) The Statistical Analysis:

The association/contingency between cervical lesion and HPV infection was statistically calculated by using the Chi-Square **(χ2)** test of significance by adopting the statistical Software Graph Pad InStat.

III. RESULT

Out of 226 cervical smears 199 pap smears were reported as NILM and 27 smears showed presence of ECA/SIL including invasive cervical carcinoma. Result of ECA category has been already reported [Sarma U.*et al*, 2013) [15]. PCR result shows 9.73% of cases to be HPV positive out of which 5.3% cases were HR HPV 16 positive, 2.6 % cases were HPV 18 positive and 1.8%

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cases were HPV positive other than HPV 16 & 18(Cite Table1). Over all HPV prevalence among ECA was 66.7% with HPV 16 being detected in 61.1% cases, HPV 18 in 27.8% cases and other subtypes in 11.1% cases respectively.

Association/contingency between cervical lesion and HPV infection was statistically calculated by using the Chi-Square ($\chi 2$) test of significance and found out to be extremely significant association among the cases between the cervical lesion and HPV criteria (Table III).

TABLE III ASSOCIATION/CONTINGENCY BETWEEN THE CERVICAL LESION AND HPV INFECTION

Pap smear	HPV +ve(22)	HPV –ve (204)	Total (226)	χ^2-Value	d.f.	Significance
SIL/ ICC	18(8%)	9(4%)	27(12%)	113.1**	1	Highly Significant (P<0.0001)
NILM	4 (2%)	195(86%)	199(88%)			

IV. DISCUSSION

In India, cervical cancer ranks first among all the female malignancies, and as observed, it is mainly associated with HPV infection. So HPV screening is one of the best ways to check if a woman is at the risk of developing cervical cancer. From Assam, very little literature on HPV prevalence is available. Therefore, we tried to evaluate the prevalence of H-R HPV 16 and HPV 18 in women attending the Gynecology OPD of Gauhati Medical College Hospital (GMCH), Guwahati by analyzing 226 paper smears from cervical specimens. Advantage of dry paper method has already been described by Kailash*etal*. The technique has been introduced in GMCH for the first time. It issimple, cheap, safe and most convenient for collection, storage and transport of cervical scrapes/smears and does not require freezing. So the samples were kept for more than a year and transported to R. M. R. C. for PCR to detect HPV DNA. The most common clinical presentation was white discharge, utero-vaginal prolapse, and cervical erosion including growth.

HPV 16 prevalence is higher in SIL/ICC cases than HPV 18 subtype and in 18.8% cases HPV sub typing could not been done as we did the tests using primers for HPV16 & HPV18 only. We have not found any single case of multiple infections. The result showed a difference of opinion while comparing to studies done by others (Cite Table2).The overall prevalence of HPV was 42.5%in the US (Susan Hariri *et al*,. 2011) [16], 13.3% in southeast China (Ye et al. 2010) [17], 15.1% in the UK (Cotton et al. 2007)[18], and 22.7% in Greek population (PetroulaStamataki*et al*.,2010) **[19].**

TABLE IVCOMPARISON OF HPV PREVALENCE IN CERVICAL CANCER IN INDIA

Regions of the study	Sample type	HPV type	Prevalence (%)	Author
Karnataka	General Population	All types	64.4	Kulkarni SS *et al*,.(2011)
		16	2.75	
		18	22.0	
		16 & 18	75.3	
	Cervical Cancer	All types	96.7	
		16	89.7	
		18	86.2	
West Bengal	General Population	All types	12.9	Laikangbam P et al,. (2007)
		16	7.80	
		18	0.90	
	Cervical Cancer	Study not done		
Sikkim	General Population	All types	12.5	Laikangbam P et al,. (2007)
		16	3.89	
		18	0.20	
	Cervical Cancer	Study not done		
Andhra Pradesh	General Population	All types	10.3	Pavani S.*et al*,.(2005)
		52	27.8	
	Cervical Cancer	16	16.7	
		18	05.6	
		All types	87.8	
		16	66.7	
		18	19.4	
Eastern India	General Population	All types	9.90	Dutta S.*et al*,.(2012)
		16	0.60	
		18	1.40	
	Cervical Cancer	Study not done		
Uttar Pradesh	General Population	All types	9.90	Srivastava S. *et al*,.(2012)
		16	63.7	
		18	3.70	
	Cervical Cancer	Study not done		
Manipur	General Population	All types	7.40	LaikangbamP et al,. (2007)
		16	3.30	
		18	2.07	
	Cervical Cancer	Study not done		
Southern Assam	General Population	Study not done		GhoshS.K. *et al*,.(2011)
	Cervical Cancer	All types	24	

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		16 18	16.7 83.3	
Lower Assam	General Population Cervical Cancer	All types 16 18 All types 16 18	9.7 5.3 2.6 66.7 61.1 27.8	Present Study

The evaluation of our study showed a lower HPV prevalence in cervical smears compared with the study done by Laikangbam P et al, (2007) [20]in Sikkim and Kulkarni SS *et al*., (2011) [21]in Karnataka and in Andhra Pradesh (Pavani*et al*,.2005) [24]but similar results were found by study done in Uttar Pradesh (Srivastava S *et al*,2012) [22] and in Eastern India (Dutta S *et al*,. 2012) [23]. In Manipur, a neighboring state of Assam, lower HPV prevalence has been observed than our study [20].From these observations it is evident that, there is quite a high percentage of HPV prevalence in the general population of Karnataka in comparison with the studies conducted in India as well as studies of global HPV prevalence. Our study is comparable to that of Uttar Pradesh. Surprisingly our data could not be correlated with that found in southern Assam. They have found higher HPV18 prevalence which contradicted our study. There may be possibility of differential distribution of HPV genotypes in different regions of Assam.

V. CONCLUSION

In spite of the low sample size, the present study showed association of HPV infection with cervical dysplasia and invasive cervical cancer with sensitivity and specificity of the study are 0.8182 (95%CI 0.5974-0.9481) and 0.9559 (95% CI 0.9178-0.9796) with positive predictive value of 0.6667(95% CI 0.4600-0.8349). A negative HPV-test result, even in combination with a positive Pap result may be LSIL, but positive H-R HPV result with a negative Pap report require follow-up. These data show that HPV testing is of value for the detection or exclusion of prevalent CIN in a routine cervical cancer-screening setting and can be used for further detecting high risk women for follow-up management. The data generated by this study may help the policy maker to include HPV testing as a routine cervical cancer screening programme.

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ABBREVIATION USED

AAR	Age adjusted incidence rate
ECA	Epithelial cell abnormality
DNA	De oxy ribo nucleic acid
PCR	Polymerase Chain Reaction
SIL	Squamous intraepithelial lesion
IARC	International association of Research on Cancer
ECA	Epithelial cell abnormality
NILM	No intra epithelial lesion/malignancy
GOPD	Gynecology outpatient department

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