RESEARCH ARTICLE

Distribution of Human Papilloma Virus Infections of Uterine Cervix among Women of Reproductive Age - a Cross Sectional Hospital-Based Study from North East India

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Abstract

Infection of the uterine cervix by human papilloma viruses (HPV) may be associated with cervical pre-cancer and invasive cervical carcinoma if left untreated. With advance in molecular techniques, it has become easier to detect the resence of HPV DNA long before the appearance of any lesion. This study concerned cervical scrape samples of 310 married non-pregnant women attending a gynecology outpatient department for both Pap and PCR testing to detect HPV DNA. Nested PCR using primers for L1 consensus gene with My9/My11 and GP6+/GP5+followed by multiplex PCR were carried out to detect HPV 16 and HPV18. Result: HPV prevalence was 11.9% out of which 3.67% cases of negative for intra-epithelial lesion or malignancy (NILM) and in 71.1% (27/38) of atypical cervical smears were HPV positive. There was increasing trend of high-risk-HPV positivity (HR HPV 16 and 18), from 20% in benign cytology (NILM) to 42.9 % in LSIL, 71.41% in HSIL and 100% in SCC. There was highly significant association of HPV infection with cervical lesion (x^2=144.0, p<0.01) and also with type specific HPV prevalence (x^2=7.761*(p<0.05).

Keywords: Human papilloma virus - cervical cancer - squamous epithelial lesion - PCR - NILM - India

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Introduction

Persistent infection with one of about 15 genotypes of carcinogenic human papilloma virus (HPV) causes progression to cervical cancer in almost all cases. Infection with virus like human papilloma virus has been implicated for invasive cervical carcinoma (Ferlay et al., 2010). Though most HPV infections are asymptomatic and cleared within 2 years; persistent genital HPV infection can lead to clinical disease including anogenital warts, cervical intra-epithelial neoplasia (CIN), cervical carcinoma and other anogenital cancers. Sexually transmitted human papilloma virus (HPV) infection is the most important risk factor for cervical intraepithelial neoplasia and invasive cervical cancer (Schiffman et al., 2007). The association of HPV infection is present in virtually all cervical cancer cases worldwide (Walboomers et al., 1999). High risk HPV types 16 and 18 are responsible for about 70% of all cervical cancer cases worldwide. The relative frequency of HPV-16/18 increases with the severity of the lesion (Carter et al., 2000). Human papilloma virus infection is measured by means of its DNA detection in cervical cells (fresh tissue, paraffin-embedded or exfoliated cells) (Rai et al., 2014).

Cervical cancer is the first kind of cancer that can be prevented through vaccination as this cancer has an infective etiology. As HPV infection spreads through sexual route, so vaccination against high risk HPV infection prior to the start of the sexual activity decreases the risk of cervical carcinogenesis. Two vaccines licensed globally are available in India; a quadri-valent vaccine and a bi-valent vaccine (Singhal, 2008). Both vaccines are manufactured by recombinant DNA technology that produces non-infectious virus like proteins comprising of the HPV L1 protein. In spite of the availability of a preventable vaccine against cervical cancer, it is the second most common cancer among women worldwide, with an estimated 529,409 new cases and 274,883 deaths in 2008 (Ferlay et al., 2010).

India, the second most populous country in the world, accounts for 27% (77,100) of the total cervical cancer deaths (Ferlay et al., 2010). The disproportionately high burden of cervical cancer in developing countries is largely due to a lack of screening that allows detection of precancerous and early stage cervical cancer (Jemal et al., 2011). India has a population of 366.58 million women ages 15 years and older who are at risk of developing cervical cancer. Any of them may develop cervical cancer. Current estimates indicate that every year 134420 women are diagnosed with cervical cancer and 72825 die from this disease. In India, cervical cancer ranks the 1st most frequent cancer in women (Human Papillomavirus

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and Related Cancers in India. Summary Report, 2010). Coming to the scenario in Assam, cancer of the cervix (AAR 16.4) is still the leading cancer among women in the Kamrup Urban district (Sharma et al., 2013). Nested multiplex PCR detected HPV in 98% cases of cervical cancer in a study carried out in a cancer hospital located in Kamrup Urbun district of Assam, a populous state in north east India (Das et al., 2013). On this background, the study was carried out with the aim to analyse the frequency of human papilloma virus infection in women of reproductive age group attending the tertiary level of medical facility and to correlate the type of cervical cellular change evoked by HPV infection.

Materials and Methods

Total 310 no. of cervical scrapings were collected from married non pregnant women aged 15-55 years during the period January'2011 till June'2013. The study was approved by institutional ethical committee vide letter No.MC/190/2007/Pt-1/71 dated 15/12/10. Prior to the collection of the specimen, the written consent from the patients attending the GOPD has been taken.

Specimen collection

Two numbers of cytological samples from each case were collected using cotton tipped applicator stick/Ayer's spatula after visualizing the cervix using the Cusco's speculum from both ecto and endo cervix including transformation zone. One cervical scrapping was placed onto clean grease free labeled glass slides for pap test, fixed immediately in absolute alcohol. The smears are stained using papanicolaou stain, examined under light microscope and reported according to the Bethesda system 2001 (Solomon et al., 2002).

The second sample of cervical scraping was smeared and dried on to Whatman 3MM filter paper and stored individually at room temperature with proper labeling. The dry paper smears were carried to Regional Medical Research Center, North East Region for detection of HPV DNA. The test was carried out according to the method described by Kailash et al. (2002) and Sotlar et al. (2004).

DNA preparation, amplification and amplicon detection

A small piece of the dried paper specimens was punched out with the help of a sterile puncher, transferred into 1.5 ml micro centrifuge tube (eppendorf, Hambarg, German,) containing 100 μl of distilled water. This way all the samples were made ready for DNA extraction. The centrifuge tubes containing the dry smear punch were boiled for 15 minutes, immediately placed on ice for 5 minutes followed by centrifuge at 10,000 rpm for 3 minutes at 4°C. 70 ul volume of supernatant kept into a fresh 1.5 ml centrifuge tube and stored at -20°C for PCR. PCR mix with Taq DNA Polymerase (Promega, USA) and the primers were added to the tube. Amplification of DNA by conventional PCR was performed in a 20μl reaction mix in a DNA Thermal cycler (Applied biosystem, thermal cycler), using primer My09/My11 which is a primer for consensus L1 gene of HPV. PCR for HPV detection was carried out using MY09/11 and GP5+/6+, HPV 16 and HPV 18 (Baay et al., 1996; Sotlar K et al,. 2004; Evans et al., 2005). Amplifications were performed with the following cycling profile: amplitaq gold activation was performed by incubation at 95°C for 5 min followed by 40 cycles of 1-min denaturation at 94°C, 1-min annealing at 55°C and 1-min elongation at 72°C. The last cycle was followed by a final extension step of 7 minutes at 72°C. After PCR, 10 μl of the amplified product were run on an ethidium bromide-stained 2% agarose gel (BioRad, USA) and visualized with a UV Transilluminator. The gel picture is photographed in a gel documentation system (GELLOGIC 2200 Image System). HPV 16 plasmid DNA served as positive controls, whereas nuclease free water served as negative control. Strict laboratory precautions and control measures were followed to avoid cross-contamination and carry-over in the PCR assay. The primer sequences used in the study are shown in **Table 1.**

Interpretation of PCR testing

The size of amplified PCR products was observed against a 100 bp DNA ladder. The band that correlated with the expected product size (450 bp), was considered positive. The all negative samples for MY09/MY11 that was observed in first round of nested PCR were further processed for 2nd round PCR using primers GP6+ and GP5+ using same PCR protocol and PCR profile and run 2% agarose gel for confirmation of results. 1 μl of the first round nested PCR amplicon was used as template for the 2nd round nested PCR. HPV sub typing was performed on positive samples using another nested PCR for the subtypes HPV 16 and HPV 18. First round PCR was

Table 1. HPV Primer Sequences Used in the Study

HPV genotype	Amplicon (bp)	Sequence (5-3)
MY09 (outer primer)	450	FP-GCM CAG GGW CAT AAY AAT GG
MY11 (outer primer)		RP-CAA CTT CAT CCA CGT TAC ACC
GP 5+	150	FP-AAT GCC TGT GTT CAT TGC TG
GP 6+		RP-TTC AAG GTC AGC CCC TAC AC
HPV 16-FP (genotype 16)	457	CAC AGT TAT GCA CAG AGC TGC
HPV 16-FP		CAT ATA TTC ATG CAA TGT AGG TGT A
HPV 18 (genotype 18)	322	CAC TTC ACT GCA AGA CAT AGA
HPV 18-RP		GTT GTG AAA TCG TCG TTT TTC A
GP-E6-3F (first round FP consensus for 16 & 18)	~630	GGG WGK KAC TGA AAT CGG T
GP-E6-5B (first round RP consensus for 16)		CTG AGC TGT CAR NTA ATT GCT CA
GP-E6-6B(first round RP consensus for 18)		TCC TCT GAG TYG YCT AAT TGC TC

*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

Table 2. Association/Contingency between Cervical Lesions and HPV Infection

HPV status Cervical	HPV+ve	HPV-ve	Total	d.f.	x^2-value	Significance
Cytology						
ECA	27 (9.0%)	11 (4.0%)	38(12.0%)	1	144	p<0.01
NLIM	10 (3.0%)	262(85.0%)	272(88.0%)			
Total	37(12.0%)	273(88.0%)	310 (100%)			

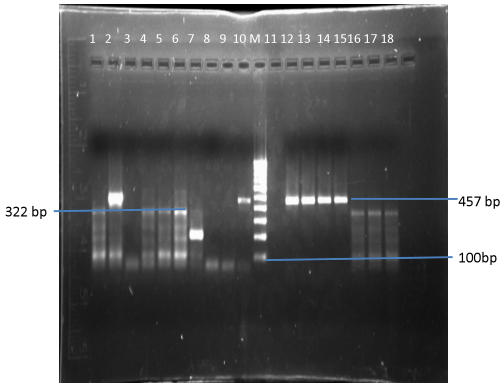

Figure 1. Post PCR Gel Electrophoresis (HPV16 at 457 bp at Lane 14, 15, 16) and HPV18 at 322bp at Lane 5, 6 7 PC at Lane 10)

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.The 2nd round of nested PCR was processed using amplicons of the first round as template in both the sets using HPV16 F andHPV16 R and HPV18 F and HPV18 R respectively. The sizes of amplified PCR products were observed against a 100 bp DNA ladder. The expected product size for HPV16 was 457 bp and 322 bp for HPV 18 (Figure 1).

Statistical Analysis

The association/contingency between demographic factors and HPV infection was statistically calculated by using the chi-square (x^2) test of significance. The HPV prevalence and the sensitivities, together with the 95% confidence intervals (CI), of the present study were estimated for subjects with cytology status of uterine cervix according to the Bethesda system 2001 (Solomon D et al., 2002). The calculations were performed by adopting the statistical software Graph Pad InStat.

Results

Thirty eight cases (12.3%) of the total study population showed evidence of epithelial cell abnormality (ECA) of varying grades. HPV related cytopathic change mostly koilocytosis in intermediate type of squamous epithelial cells were noted in 18.4% smears and termed as low grade squamous intra-epithelial lesion (LSIL). High grade squamous intra-epithelial lesion (HSIL) was found in 28.95% smears. Evidence of squamous cell carcinoma features were noted in 31.58% smears. In 21.05% smears were categorized under atypical squamous cell of undetermined significance (ASCUS) group as there were very few atypical squamous cells some showing cytoplasmic cavitation, yet they could not be graded into squamous intra epithelial lesion.

Over all HPV prevalence in general population was 11.94% (37/310). HPV DNA was detected in 3.67% (10/272) cases of NILM (Negative for intra-epithelial lesion or malignancy) cases and in 71.05% (27/38) of total ECA out of which 51.85% (14/27) of HPV positive ECA cases were HPV16 positive and 18.5% cases were HPV 18 positive. It was 62.5% in patients with ASCUS, 100% in patients with LSIL, 63.6% in patients with HSIL and 66.67% in patients with squamous cell carcinoma. Similarly, there was increasing prevalence of high-risk-HPV 16 and 18 sub-types, from 20% in benign cytology (NILM) to 42.9% in LSIL, 71.41% in HSIL and 100% in squamous cell carcinoma. HPV 16 viral DNA were detected in 68.75% of higher grade cervical lesion i.e. both high grade squamous intra-epithelial lesion and squamous cell carcinoma in 12 cases, the HPV sub typing could not been done as the tests were done using primers for HPV16 and HPV18 only. The HPV status of all the cases is detailed in Table 1.

There was an increasing trend of HPV prevalence of high-risk-HPV 16/18 sub-types, from 20% in benign cytology (NILM) to 42.9 % in LSIL, 71.41% in HSIL and 100% in SCC. It has been observed that there is highly significant association of HPV infection with cervical lesion (x^2=144.0, p<0.01) (Table 2). Significant association is also noted when data is compared with type specific HPV prevalence (x^2=7.761*p<0.05). The sensitivity of the study with respect to HPV prevalence is 0.7297 (95% CI: 0.5584 to 0.8620) and specificity is 0.9597(95% CI: 0.9290 to 0.9797)

Discussion

In the present study, benign pathology (NILM) was detected in 87.7% of the total cases out of which 3.67% cases had HPV infection. Cervical intra epithelial neoplasia and squamous cell carcinoma (ECA) were detected in 38(12.3%) numbers of cervical scrapings of the total participants in the study population. Out of them, 71.05% (27/38) cases had HPV infection. In 28.95% (11/38) cases, HPV DNA could not be detected. Low viral copy or sampling error may be the possibility for HPV negativity in these cases. Park et al. (1994) could not detect HPV infection in 15.17% (39/257) cases of cervical cancer specimen even by Southern blot analysis and polymerase chain reaction. Generally scraped cervical cells are collected in cold PBS or kit-specific solutions and transported on ice, requiring constant refrigeration. But in the present study, specimens were collected in 3 MM Whatman filter paper for DNA extraction instead of liquid based solution (PBS). For DNA extraction, boiling

method was carried out to extract viral DNA from the dry paper smear as described by Kailash et al. (2002). This method had been applied in this institute (GMCH) for the first time for detection of viral DNA. So the samples were kept for more than a year at room temperature and transported to regional medical research center for North-East (ICMR), dibrugarh for PCR testing to detect HPV DNA. Boiling method was applied to extract DNA from the dry paper smear. The outcome of the study that had used the application of Paper smear technique showed a satisfactory result and tallied with other studies (Kailash et al., 2002; Shukla et al., 2010).

The study of Sufang et al. (2005) showed that positive rates for HPV DNA in normal population and cervical cancer patients were 32.99% and 73.53% respectively. The present study also reported similar results with 71.05% HPV positivity in cervical cancer and CIN. Cotton et al. (2007) had found that high-risk HPV prevalence was increased with increasing smear grade. The result of Elena Argyri et al. (2013) showed a significant association of HR HPV prevalence with epithelial cell abnormality detected in cervical smears. The present study also showed a strong relationship between HR HPV types and higher grade cervical lesion. Argyri et al. (2013) had found out that HPV 16 infection was detected in 14.5% of LSIL and 37.2% of HSIL category respectively. The present study too detected HPV 16 viral DNA in 33.3% (2/6) of LSIL, 37.5% (3/8) of HSIL and 100% (8/8) of SCC respectively. Study of Kitchener et al. (2006) showed that the proportion of women with HPV 16 infections increased from 1.5% in women with normal cytology to over 50% in those with severe dyskaryosis. They concluded that as the grade of cytological abnormality increased, the proportion of high risk HPV positivity also increased. Similar results were found in the present study with 3.67% HPV positivity in benign lesion to 71.03% in those having cervical lesion of varying grades. High risk HPV 16 viral DNA were detected in 66.7 % of the total squamous cell carcinoma included in the present study. The largest meta-analysis studied by Bruni et al. (2011) had detected 11.7% estimated global HPV prevalence (95% CI-11.6-11.7%); most common type specific HPV was HPV 16 with its prevalence being 3.2% and HPV 18 being 1.4%. The result of overall HPV prevalence found in the present study is comparable to that done by Bruni et al. (2011) but a difference of opinion while comparing to type specific HPV 16 and HPV 18. The present study showed that the HPV 16 prevalence was 4.8% and HPV 18 being 1.9%. In spite of lower HPV prevalence (8.8%) detected by study of Dunne EF et al. (2011), found out type specific HPV16 and HPV18 prevalence with 4.7% (95% CI; 4.0%-5.5%) for HPV 16 and 1.9% (95% CI; 1.4%-2.5%) for HPV 18. The present study regarding HPV 16 and HPV 18 also revealed similar result. According to UICC board of directors, 50 to 60% of squamous cell carcinoma of the cervix are caused by HPV 16 infection. Detection of HPV in cervical cancer specimens from different parts of India indicates that HPV 16 is the most predominant type (Das B et al., 2000). Study of Lin et al. (2001) also detected 64% of the cervical cancer patients tested positive for HPV at the time of enrollment into the study.

The present data on HPV prevalence in the cytology specimens showed discrepancy to the study carried out by Walboomer et al. (1999) who established the presence of HPV in virtually all cervical cancers. Disruption of the L1 region of HPV DNA (which is normally used for HPV detection and typing) may account for false-negative or undetectable HPV test results among cervical cancer cases (Herrington C et al., 1999; Walboomer et al., 1999). The possibility for HPV negativity in these cases may be due to problems arising from cervical samples which were collected in Whatmann 3 MM filter paper; the yield of DNA per disc obtained from the paper smear was found to be relatively low compared with that in liquid-based collection and was a limiting factor for doing multiple reactions and experiments. However HPV 16 was more common in higher grade lesion.

The present study concluded that the data generated by the study are cross sectional in nature and cannot distinguish incident from prevalent infection. Moreover, the evaluations are based on HPV DNA testing which, although it is the best indicator of current infection, is not a measure of cumulative exposure, because HPV DNA may clear or become undetectable in previously infected individuals. Therefore, the prevalence estimates do not reflect lifetime risk of HPV infection. Though cervical cytological study with help of pap stain is the most widely available tests for detection of HPV related change including CIN and cervical cancer, cytology alone may not predict the HPV status if the change at tissue level yet to develop. However, with advancement in newer molecular technique, it becomes easier to detect presence of HPV DNA from a patient long before the appearance of the lesion. HPV testing is of great value for the detection or exclusion of prevalent CIN in a routine cervical cancer-screening setting, thereby provides many opportunities for detection and treatment of the precancerous condition, often with high cure rate. Such type of study may help the policy maker to include HPV testing as a routine cervical cancer screening programme. The present study emphasizes the necessity of confirmation of HPV presence in cytological specimens by a more sensitive and specific molecular technique such as the PCR. The persistence or progression to high grade lesions in patients with mild cytological abnormalities can be predicted by molecular detection of HPV in some cases, particularly when combined with cytological analysis.

The accurate detection of an infectious agent (HPV) in a specimen will provide consistent and meaningful result in research and clinical setting to help target and focus resources in disease prevention and control. It is the right time for medical societies and public health regulators to consider this evidence and to define its preventive and clinical implications

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