Original Article

Prevalence of high - risk human papillomavirus infections in women with benign cervical cytology: A hospital based study from North India

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Abstract

INTRODUCTION: Cervical cancer is the commonest cancer among Indian women. High-risk human papillomavirus (HPV) detection holds the potential to be used as a tool to identify women, at risk for subsequent development of cervical cancer. There is a pressing need for identifying prevalence of asymptomatic cervical HPV infection in the local population. **OBJECTIVE:** To determine the prevalence of high–risk HPV DNA in women with benign cervical cytology. **MATERIALS AND METHODS:** Women visiting the gynecology outpatient with varied complaints were subjected to Pap smear. Four hundred and seventy two samples were subjected to polymerase chain reaction, using consensus primers for low and high-risk HPV (types 6, 11, 16, 18, 31 and 33). The samples that were positive for HPV DNA were subsequently assessed for high-risk consensus primers, types 16, 18, 31 and 33 as well as for HPV type 16 and 18. **RESULTS:** One hundred and seventy four (36.8%) women tested positive for HPV DNA. Thirty nine (8.2%) of the entire cohort tested positive for high-risk HPV. Fifteen samples were positive for type 16, 22 for type 18 and two for both types 16 and 18. A statistically higher prevalence of high-risk HPV was observed in poorly educated and rural groups. No association of HPV prevalence was noted with age, parity and age at marriage. **CONCLUSION:** The study generates epidemiological data of prevalence of sub-clinical HPV in the women visiting a tertiary care institute as well as peripheral health centres. The data generated will be useful for laying guidelines for mass screening of HPV, treatment and prophylaxis in the local population.

Key words: Human papillomavirus, polymerase chain reaction

Introduction

Human papillomavirus (HPV) is a widely prevalent sexually transmitted virus.[1,2] Although the majority of infections are benign and transient, persistent infection is associated with the development of cervical and other anogenital cancers.[3,4] Cervical cancer is the commonest cancer among Indian women. Approximately 20,000 new cases were detected in India, in the year 2000.[5] HPV infection is typically asymptomatic to begin with.[6] The transmission occurs prior to any clinically detected expression of the virus. HPV infects the basal cells of the epithelium.[7] The virions assemble in the nucleus and are subsequently shed from keratinocytes. There is proliferation of all the epithelial layers, except the basal. The virus has an incubation period of 3-4 months.[8] It clinically manifests as hyperplastic, hyperkeratotic warts or dysplastic lesions that may undergo neoplastic transformation.[9] Based on the epidemiologic classification of HPV types by Munoz *et al*, the high-risk types are 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82. Low–risk types are 6, 11, 40, 42, 43,

44, 54, 61, 70, 72, 81 and CP 6108.[10]

It is now universally accepted that nearly all the invasive cervical cancers and high-grade intraepithelial neoplasias are associated with the high-risk HPV types. Owing to the strong association, it has been suggested that high-risk HPV detection might be used as a tool to identify women at risk for the subsequent development of cervical cancer. Guidelines need to be formulated for HPV testing in cervical cancer screening and for vaccination. For this, age related prevalence of high-risk HPV in cytologically normal cervical smears, needs to be determined. With this aim, we undertook the present study, to estimate the prevalence of high-risk HPV using highly sensitive polymerase chain reaction (PCR).

Materials and Methods

Patients and cervical smear

Papanicolau (pap) smear cell samples were obtained randomly from new patients attending the gynecology outpatient clinic of a tertiary care hospital, general hospital and from a peripheral health centre ie a dispensary, for varied complaints. Pregnant women and women with a history of hysterectomy or conisation were excluded. Subsequently, patients in whom the cervical cytology was found to be atypical, were excluded from further analysis. Relevant data, viz; age, parity, chief complaints, clinical diagnosis, examination findings, etc., were recorded on a pre-designed performa by a postgraduate medical doctor, exclusively on roll for the study. Cervical scrape smears were obtained by Ayers spatula. The tip of the spatula containing the sample was rinsed in a self-standing centrifuged tube containing 1 x phosphate buffered saline (PBS, pH 7.4).

DNA extraction

DNA was isolated using a standard protocol.[11] The exfoliated cells were pelleted out. The cell pellet was resuspended in Tris –EDTA buffer (pH 8.0) and treated with 10% sodium dodecyl sulphate and 10 mg/ml proteinase K (Roche, Germany) at 65°C, for one hour. DNA was extracted using phenol-chloroform-isoamyl alcohol mixture (25:24:1 v/v) and precipitated with isopropanol. The quantity of DNA was estimated spectrophotometrically. PCR for β actin gene was also performed for each sample, as an internal control. The DNA samples which did not show PCR product with the same were excluded.

PCR for HPV

PCR was performed on extracted DNA using primers from consensus sequence, spanning the E1 open reading frame of the HPV genome,[12] to detect types 6, 11, 16, 18, 31 and 33. The sequence of sense primer was 5'- TATGGCTATTCTGAAGTGGAA-3' and that of anti-sense primer was 5'- TTGATATACCTGTTCTAAACCA-3'. The reaction was carried out in a volume of 20 µl, containing the following: 2 µl 10X Taq buffer, 2 mM Magnesium Chloride, 5.0 pmol of each of the sense and anti-sense primers (Sigma, USA), 250 µM dNTP mix, 2.0 units Taq polymerase (Invitrogen, USA) and sterile distilled water. Three microlitres of template DNA were added to each reaction. The plasmid DNA for HPV types 6, 11, 16 and 18 were used as positive control in the reaction. Reaction was performed in a DNA thermal cycler (Eppendorf, Germany) as per the understated protocol.

Denaturation was done for 10 minutes at 94°C for the first cycle. This was followed by one minute each of denaturation at 94°C, annealing at 46°C and extension at 72°C, for 33 cycles. The last cycle was extended for 10 min at 72°C. The electrophoresis of amplified products was done in 1.5% agarose gel. The gel was stained with ethidium bromide, to visualize the amplified PCR product. A 526-594 base pair (bp) band was visualized in the positive samples for HPV on a UV transilluminator.

Type specific PCR for HPV 16 and18

The positive samples were subjected to PCR using a pair of oligonucleotide primers, specific for consensus sequence, spanning the E6 open reading frame of high-risk HPV types 16, 18, 31 and 33.[13] The sequence of forward primer was 5'-TGTCAAAAACCGTTGTGTCC-3' and that of reverse primer was 5'-GAGCTGTCGCTTAATTGCTC-3'. Positive samples thus obtained were subjected to type specific PCR for HPV types 16 and 18.[14] PCR was performed using type specific primers for HPV 16. The sequence of forward primer was 5'-ATTAGTGAGTATAGACATTA-3' and that of reverse primer was 5'-GGCTTTTGACAGTTAATACA-3'. The forward and reverse sequence of HPV type 18 specific primers, was 5'-ACTATGGCGCGCTTTGAGGA-3' and 5'-GGTTTCTGGCACCGCAGGCA-3', respectively. The generated fragments were of 109 bp and 334 bp for HPV 16 and 18, respectively and were visualized on 2% agarose gels.

Statistical analysis

Statistical analysis has been performed with the Fisher exact test; *P* value less than or equal to 0.05 is taken as significant.

Results

Pap smear was obtained after an informed consent from

509 women. Eleven (2.3%) samples yielded DNA of unacceptable quality and were excluded. Twenty six (5%) smears had atypical cells on cervical cytology and were excluded from further analysis for the present study. Further discussion will be restricted to the remaining 472 women.

The mean age was 37.5 ± 11.3 years (range: 19-75). Twelve women (2.5%) were on oral contraceptives. Six (1.2%) smoked. Genital warts were not documented on clinical examination in any subject. One hundred and eighty one (38.3%) pap smears were found to be inflammatory. High-risk HPV was observed in 18(10%) inflammatory smears, whilst 21 (7.2%) smears with normal morphology were HPV positive (P= 0.075). One hundred and seventy four (36.8%) women tested positive for HPV DNA (Types 6, 11, 16, 18, 31 and 33) by PCR, using consensus primers spanning the E1 ORF [Figure 1]. One hundred and one (58%) of these positive women, complained of vaginal discharge and were subsequently diagnosed to have monilial, trichomonal or mixed vaginitis. Thirty-nine (8.2%) of the entire samples tested positive for high-risk HPV (types 16, 18, 31 and 33), using consensus primers, spanning the E6 ORF.

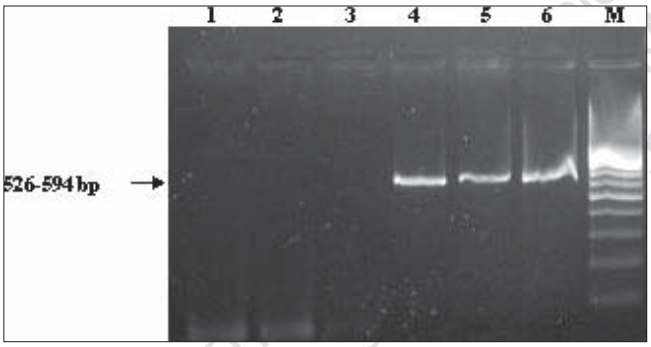

Figure 1: Agarose gel electrophoresis of PCR products of HPV types 6, 11, 16, 18, 31 and 33 using consensus primers (526-594 bp). Lane 1: negative control, lanes 2 and 3: negative samples, lane 4 and 5: positive sample, Lane 6: positive control and M: DNA marker (100bp ladder)

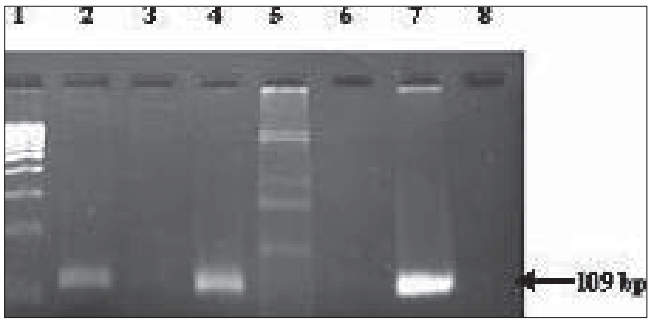

Figure 2: Agarose gel electrophoresis of PCR products of HPV type 16 (109 bp). Lane 1: DNA Marker (100 bp ladder), lane 2 and 4: positive samples, lane 3, 5 and 6: negative samples, lane 7: positive control and lane 8: negative control

All samples that were positive for high-risk consensus primers (types 16, 18, 31 and 33), tested positive with type specific primers for HPV type 16 or 18 or both 15(3.2%) tested positive for type 16 [Figure 2], 22(4%) for type 18 and two (0.4%) for both 16 and 18. The prevalence of HPV DNA, according to age, age at marriage and parity is illustrated in Table 1. Association of other variables with HPV DNA positive cases is depicted in Table 2.

Discussion

HPV is recognized as a public health problem for its role as a critical factor in pathogenesis of various cancers. Cervical cancer is a preventable disease [15]. It develops following progression of uncleared HPV infection to high grade and eventually to invasive disease.[16] Women with normal cervical cytology, who are infected with high-risk HPV, have an approximately 100-fold increased risk of developing CIN 3, compared with uninfected women.[17] Persistence of oncogenic human papillomavirus appears essential for the development of cervical neoplasia.[16] With the advent of molecular techniques, particularly PCR, it is possible to detect very low quantities of HPV and to subtype the commonly occurring HPV in cervical scrape smears. The cytologic features of HPV on Pap smear are non-specific.

Table 1: Demographic characteristics of women, correlated with high-risk HPV DNA prevalence

Age (yrs)	No. of women	No. of women positive for HPV DNA*(%)	No. of women positive for HPV 16 or 18 or both (%)
< 30	190	74 (38.9)	15 (7.8)
30-40	168	62 (36.9)	12 (7.1)
40-50	80	29 (36.25)	11 (13.7)
≥50	34	11 (32.3)	1 (2.9)
Age at marriage (yrs)			
<16	59	24 (40.6)	7 (11.8)
16-20	230	80 (34.7)	20 (8.6)
20-24	134	58 (43.2)	7 (5.2)
24-28	42	14 (33.3)	5 (11.9)
≥28	7	4 (57)	nil
Parity			
0	67	29 (43.2)	4 (5.9)
1	50	17 (34)	3 (6)
2	133	41 (30.8)	8 (6)
3	102	30 (29.4)	11 (10.7)
>3	120	57 (47.5)	13 (10.8)

*Positive for HPV types 6, 11, 16, 18, 31 and 33 (using consensus primers), HPV - Human papillomavirus

Table 2: Distribution of high-risk HPV DNA cases according to various variables

Cervix	No. of women	No. of women positive for HPV DNA* (%)	No. of women positive for High-risk HPV16 or 18 or both (%)
Healthy	321	127 (39.5)	24 (7.4)
Erosion	109	36 (33)	9 (8.2)
Cervicitis	42	11 (26.1)	6 (14.2)
Socio economic (Rs/per year)			
Low (10,000)	59	37 (62.7)	7 (11.8)
Middle (>10,000-40,000)	211	68 (32.2)	18 (8.5)
High (>40,000)	202	75 (37.1)	14 (6.9)
Literacy (yrs of education)			
Illiterate or <6	160	75 (46.8)	21 (13.1)
≥6-9	64	17 (26.5)	6 (9.3)
>9-12	149	50 (33.5)	8 (5.3)
Graduate	99	32 (32.3)	4 (4)
Residence			
Urban	297	95 (31.9)	16 (5.3)
Rural	175	79 (45.1)	23 (13.1)

Positive for HPV types 6, 11, 16, 18, 31 and 33 (using consensus primers), HPV - Human papillomavirus

The conventional Pap smear has restricted value in identifying women, destined to develop cervical neoplasia.[18] The ALTS study (Atypical squamous cells of undetermined significance – Low grade squamous intraepithelial lesion triage study), aimed at resolving the issue of management of low-grade cervical lesions. They concluded that women with less than cervical intraepithelial neoplasia 2 (CIN 2) status at initial colposcopy remain at risk for subsequent CIN 2 +. Follow–up HPV testing is significantly more sensitive than cytology (P= 0.015) for detecting missed prevalent cases. In the same study, few cases of CIN 3 had a negative HPV test, which reinforcing, that even the most sensitive test cannot provide perfect assurance. Thus HPV testing should be used as an adjunct to Pap smears.[19]

There is ample data on prevalence of HPV in women with cervical cancer, however data on HPV prevalence in women with clinically normal cervix from India is sparse [Table 3]. The prevalence of HPV using consensus primers for types 6, 11, 16, 18, 31 and 33 among women with benign cervical cytology in the index study was 36%. High-risk HPV types 16 and 18 were detected in 8.2% of the entire sample and in 22% of the samples positive for HPV DNA types 6, 11, 16, 18, 31 and 33, using consensus primers. The prevalence of HPV 16/18 in the index study, is consistent with that reported by Duttagupta *et al*.[20] Probable incrementing factor for the high prevalence of HPV DNA is poor hygiene. It is corroborated by the observation that a significant number, i.e., 58% of positive women had vaginitis. Poor hygiene was noted to be associated with a higher prevalence of HPV in women in the control group by Franceschi *et al*,[21] as well. Women who did not toilet their genitals after intercourse or during menstruation have been found to be at a greater risk.[22] Women using homemade pads during menstruation have been shown to have a 3 to 4 fold increased risk of cervical cancer.[20]

Women who were illiterate or had less than six years of education had a significantly higher rate of high-risk HPV (P=0.014) in the index study. High-risk HPV was more common in rural than the urban women and the difference was statistically significant (P=0.001). Women belonging to low socioeconomic class had a higher rate of high-risk HPV infection, than those from medium or high socioeconomic group, although the difference was not statistically significant. Franceschi *et al*,[21] has recognized low socioeconomic status as a risk factor for cervical carcinoma as well.

No significant age related difference was noted in the index study in the distribution of HPV. Duttagupta *et al*[20] made similar observations of HPV16/18 prevalence among Muslim women. Chaouki *et al*[22] reported similar findings. More number of women (10.8%) with three or more children, were positive for high-risk HPV as compared to those with less than three children (6%). The difference was however not statistically significant. Duttagupta *et al*[20] and Lazcano *et al*,[23] too did not observe any significant association of HPV 16/18, with parity.

The socio cultural stigma of the conservative Indian society plays an important role in the reporting of promiscuity. Hence, due to the possibility of underestimation of this sensitive parameter, age at first intercourse was not elicited and age at marriage was recorded instead. No significant difference of HPV distribution with age at marriage was detected. Duttagupta *et al*,[20] similarly, did not find any association of HPV 16/18 with age of consummation of marriage.

In the index study, two types of high-risk HPV for

Table 3: Review of previous studies in women with normal cervical cytology

Reference	Place	Subjects enrolled	Number	Positivity	Method
Kulasingam *et al* (2002)[27]	U.S.A	Annual examination at planned parenthood clinic (18-50 yrs)	3318	12% of HR HPV	PCR
Chaouki *et al* (1998)[22]	Morocco	General hospital and cancer hospital (18-70 yrs)	185	20.5%	PCR
Womack *et al* (2000)[28]	Zimbabwe	Primary health clinics (25-55 yrs)	556	39%	Hybrid capture
Sellors *et al* (2000)[29]	Canada	Cervical screening from health planning regions of Ontario (15-49 yrs)	878	10.2%	Hybrid capture and PCR
Maehama *et al* (1999)[30]	Japan	Annual cervical screening (17-85 yrs)	4089	10.7% HPV DNA	PCR
Thomas *et al* (2004)[31]	Nigeria	Overall population >15 sexually active women	932 (all enrolled subjects)	24.8% in women without cervical lesion	PCR and southern blot
Ekalaksananan *et al* (2001)[32]	Thailand	Asymptomatic women attending Obes –Gynae out-patient	174	21% (Type 6 and 11)	PCR
Wickenden *et al* (1987)[33]	U.K	Women attending family planning clinic, STD clinic, F-U clinic after treatment of CIN	215	10% (HPV 16)	Dot-blot hybridization
Oh *et al* (2001)[13]	Korea	Women attending general health clinic (23-72 yrs)	1144	0.7% (high-risk HPV DNA)	PCR
Rolon *et al* (2000)[34]	Paraguay	Out patient clinic of cancer hospital and clinical hospital in Asuncion	101	20% (HPV) 5.5% (HPV 16)	PCR
Molano *et al* (2002)[35]	Colombia	National cervical cancer control programme (18-85 yrs)	1859	14.9% HPV DNA 9% HR HPV DNA	PCR
Lazcano-ponce *et al* (2001)[23]	Mexico	Morles state household sampling frame	1248	14.5% HPV DNA	Reverse line blot strip assay and PCR
Clifford *at al* (2005)[24]	Multicentric study	Women randomly selected from the general population 15-74 yrs)	15,613	1.4-25.6% HPV DNA	(PCR – based EIA

HPV - Human papillomavirus, PCR - Polymerase chain reaction

subtyping have been included, as they are more prevalent in this part of the world [Table 4]. Types of HPV in primary screening depend on the population being screened, due to the differences in prevalence of HPV types. Clifford *et al*[24] have suggested that cost-effective test could include subset of high-risk HPV, which are most likely to progress to cancer. We observed high-risk HPV DNA in 8.2% of women. The figure reported is low as compared to previous studies reported from India[17,25,26] [Table 4]. This can plausibly be attributed to the fact that previous researchers have targeted women in the high-risk groups, viz, women from rural background or those from low socioeconomic background.

The index study generates epidemiological prevalence data of sub-clinical high-risk HPV infection. The subjects were enrolled from both peripheral health care centres as well as tertiary hospital in order to include patients from all sections of the society. We had observed a lower prevalence (2.3%) (Data not shown) of high-risk HPV, initially when the subjects were recruited from the tertiary care centre alone, in the beginning of the study. This was in all probably due to better socioeconomic status and literacy of the patients. The limitation of the index study is that, being an hospital-based study, the women enrolled were not truly healthy, as they visited the hospital with varied ailments and thus were not a true representation of the community. In addition, prevalence of high-risk HPV, other than HPV 16 and 18 was not evaluated.

Cervical cancer screening practices are inconsistent in India. Use of Pap smear, as a sole indicator for screening has limitations. The cytological interpretation

Table 4: Review of previous studies from India

Authors	Place	Study design	Method	Prevalence
Franceschi *et al* (2003)[17]	Chennai	Admitted patients and their visitors of cancer institute taken as controls n=184	PCR and southern blotting	27.7% (HPV DNA) 16.32% (HPV 16 and 18)
Duttagupta *et al* (2002)[20]	West Bengal	Rural women from low socioeconomic families attending reproductive and child health clinic n=792	PCR	8.8% (HPV 16/18)
Gopalkrishna *et al* (2000)[25]	New Delhi	Women attending hospital outpatient for birth control or health check up n=30	PCR	13.3% (HPV 16)
Sarnath *et al* (2002)[26]	Mumbai	Women attending radiotherapy department of Tata Memorial Hospital with clinically normal cervix hospital n=164	PCR and southern blotting	15.2% (HPV 16/18)
Clifford *et al* (2005) [24]	Tamil Nadu	Women randomly selected from the general population n= 1179	PCR- based EIA	14.2% (HPV DNA)
Present study	Chandigarh	Hospital based study of women with benign cervical cytology n=472	PCR	36.8% (HPV DNA) 8.2% (HPV type16/or 18)

PCR - Polymerase chain reaction, HPV - Human papillomavirus

becomes faulty if the smear is inflammatory; a situation not infrequent among women from low socio-economic background. In a scenario of infrequent screening, screening with a test of high sensitivity provides greater reassurance, that potential disease has not been missed in women who screened negative. It is an irony that middle and high socioeconomic women, who can afford HPV screening by molecular techniques, require it the least, owing to the low prevalence. High-risk HPV DNA screening appears to be a valid option in mass cervical screening programmes in developed countries. In a resource poor country, it is not feasible to offer universal molecular testing for high-risk HPV, till HPV screening is made cheaper. Identification of population at risk will enable focused screening, with a greater cost effective utilization of resources. Index study has identified illiterate women and those from rural and low-socioeconomic background to be at a greater risk for HPV. Screening can preferentially be directed to the target population for optimal utilization of resources. Needless to say, health education, promotion of condom usage and need to follow healthy hygienic practices is the most cost- effective approach in reducing the incidence of cervical carcinoma in resource- crunched societies.

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