RESEARCH PAPER | Medical Science | Volume : 5 | Issue : 7 | July 2015 | ISSN - 2249-555X

Human Papillomavirus Detection in the Women of Jammu Region of Jammu and Kashmir: A Hospital Based Study.

KEYWORDS: Human Papillomavirus, Polymerase chain reaction, Jammu and Kashmir

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ABSTRACT *Background: Cervical cancer is most common malignancy in females in the age group of 25-65 years and is also the commonest cause of female death. Human papillomavirus (HPV) has been identified as the significant risk factor for this cancer. Therefore, the present study is designed to check the prevalence of HPV and HR-HPV 16, 18, 31 & 33 infections in the women of Jammu region of J&K state.*

Methods: Cervical tissue samples (exfoliates) of 500 women, attending OPD of different hospitals of Jammu region with varied complaints, were collected and were screened for the presence of HPV genome by using polymerase chain reactions (PCR). Samples found positive for HPV were further analyzed for pinpointing the specific type of high risk HPV therein by using type specific primers for HPV 16, 18, 31 & 33.

Results: Out of 500 samples 40.8% were found to be positive for HPV DNA. Among them high risk HPV-16 and HPV-18 was the most common and observed in 9.2% samples followed by HPV-33 and HPV-31 (1% and 0.8% respectively). Higher HPV percentage was observed in women with >65 years of age (71.53%), married before 18 years of age (48.44%) and with 4 or more children (50%). The study is first of its kind in Jammu region and the results have been compared with the available data on North Indian females reported by various workers.

Conclusions: The study shows high prevalence of high-risk HPV DNA in cervical tissue of women of the Jammu region of J&K State. The data generated will be useful for laying the future guidelines for molecular genetic screening and treatment of HPV infected women in the state.

Introduction:
Changes in cervix are often caused by a virus known as Human Papilloma Virus (HPV). This viral infection is recognized as a major health problem for its critical role in pathogenesis of various cancers. There is a strong relationship between the presence of human papillomavirus (HPV) and squamous lesions of the female and male genital tract. The contribution of human papillomavirus (HPV) infection to the pathogenesis of cervical cancer is well established (Schiffman et al., 2007). Cervical cancer is the second most common cancer, after breast cancer, in the women worldwide (Shanta et al., 2000; Schiffman et al., 2007).

More than 200 different HPV types have been known to occur and approximately 80 out of these have been well characterized (Forbes et al., 2007). Out of these approximately 30 are responsible for warts, lesions and cancers of genital tract in humans. On the basis of the severity of the disease caused by these types of HPV, these are classified by Munoz et al., 2003 into High risk HPV types like 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, 82 and low risk HPV genotypes like 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81 and CP 6108.

Globally, there are approximately 530,000 new cases of cervical cancer with an annual mortality rate of 275,000 (Jemal et al., 2011) and in India approximately 134,420 women are diagnosed with the disease and out of them 72,825 died every year (According to World Health Organization,2012).

The risk of developing cervical cancer is more in normal women who is infected with High risk HPV as compared to normal uninfected women or women infected with Low risk HPV. Therefore, detection of HR-HPV might be used as a tool to identify women at high risk of developing cervical cancer, in addition to pap smears analysis.

Since the lifelong risk of HPV infection in women is 80% and only a small proportion of women infected with HPV develop high grade cervical neoplasia and in the rest, HPV, disappear spontaneously without clinical lesion. Only persistent HR-HPV infection induces higher risk for the development of a high grade precancerous lesion or cervical cancer (Walboomers et al., 1999 and Vizcaino et al., 2000).

Therefore, it is important to identify those HPV infected women, who are at an increased risk of developing cervical carcinoma, by methods that reveal persistent HPV infection or detect viral oncogene expression and investigation of additional human markers for cervical carcinogenesis (Sotlar et al., 1998, Syrjanen & Syrjanen, 2000 and Moberg et

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al., 2003).

With this aim, we undertook the present study for the very first time in the women of Jammu region of J&K state, to estimate the prevalence of HPV and its genotypes by using highly sensitive Polymerase Chain Reaction (PCR) technique.

Methods:

Patients

Married and non-pregnant women without a history of hysterectomy or conisation were the subjects of the study. The study subjects were explained about the purpose of the study and informed written consent duly signed by each subject was obtained. Relevant information regarding age, parity, chief complaints, clinical diagnosis, examination findings, etc. were recorded on a pre-designed questionnaire. Study plan and questionnaire was duly approved by Animal and Human experimentation ethical committee (AHEEC), University of Jammu.

Cervical smear

Cervical tissue (Exfoliates) samples were obtained from the posterior vaginal pool by special endo-cervical brushes. These brushes along with the collected tissue were then rinsed in a self-standing centrifuged tube containing 1% Phosphate Buffer Saline (PBS) with pH 7.4 and stored at -20°C for further analysis. The samples were collected from the women with some gynecological complaints attending the Out Patient Department of Gynecology and Obstetrics, Shri Maharaja Gulab Singh Hospital (SMGS), Govt. Medical College (GMC) Jammu and other district hospitals of Jammu region of J&K state.

DNA extraction

Suspension of the exfoliated cervical cells in lysis buffer (10 mM Tris-HCl, pH 7.5, 5 mM EDTA, pH 7.9, 1% SDS) was treated with proteinase K (200 mg/ml) overnight at 37°C. DNA was extracted by using phenol-chloroform-isoamyl alcohol mixture (25:24:1) extraction and precipitated with ethanol (Sambrook and Russell, 2001) with little modifications. Quantity and quality of DNA was ascertained by spectrophotometrically and agarose gel electrophoresis, respectively.

Polymerase Chain Reaction (PCR) for HPV detection

All DNA samples were subjected to Polymerase Chain Reaction (PCR) by using consensus primers (table no.1:Primer sequences used for the detection of different HPV genotypes) to confirm the status of broad range of HPV types by amplifying a fragment of 240bp derived from the E6 open reading frame. Each amplification reaction was carried out in a total volume of 25µl containing 10mM Tris-HCl, pH 8.3, 50mM KCl, 1.5mM $MgCl_2$, 200mM of each of dNTP, 100p moles of each primer, approximately 500ng of specimen DNA and 2.5 unit of thermostable DNA Taq polymerase.

Reaction was performed in the machine, DNA Thermal cycler (Applied Biosystems, USA) as per the given protocol as under;

Initial denaturation at 94°C for 3 minutes followed by 35 cycles with denaturation for 1 minute at 94°C, annealing at 55°C for 1 minute and extension at 68°C for 2 minute and a final extension at 72°C for 1 minute.

The PCR products were electrophoresed in 2% agarose gel stained with Ethidium Bromide (Maniatis et al. 1982). The amplified products were visualized under UV transilluminator. The specimens showing PCR amplification products of the size 240bp (Fig. 1: Gel image showing PCR product of HPV consensus primer) were considered to be HPV positive specimens.

Type specific PCR for High-risk HPV detection

HPV positive samples were subjected to further PCR using type specific primers for HPV types 16, 18, 31 and 33. The sequence of the primers used with their product size is given in table no. 1(Primer sequences used for the detection of different HPV genotypes.)

PCR conditions for these HR-HPV primers were, denaturation was carried out at 95°C for 3 minute followed by 30 cycles with denaturation at 95°C for 1 minute. The annealing conditions for HPV 16 and 31 were 55°C for 1 minute and for HPV 18 and 33 were 54°C for 1 minute. The final extension cycle for HPV 16, 18, 31 and 33 were performed at 72°C for 1 minute. The amplified PCR product show band size of 109bp (Fig. 2) for HPV-16, 334bp (Fig-3) for HPV-18, 613bp (Fig-4) for HPV-31 and 411bp (Fig-5) for HPV-33 when loaded (10µl) and ran at 90 volt for an hour in 2% agarose gel and images were documented. Each amplification reaction mixture for total volume of 25 µl contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM of each of dNTP, 100 p moles of each primer, approximately 500 ng of specimen DNA and 2.5 unit of taq DNA polymerase.

Results:

Out of 500 cervical exfoliate samples of the suspected women, HPV DNA was detected in 204 (40.8%) cases and were designated as positive cases for HPV infection. These positive cases were then subjected to genotyping, so as to know the infective strains of HPV by using type specific primers and polymerase chain reactions (PCR) technique for the detection of HPV-16, 18, 31 and 33 types.

55 cases were found positive for four HR-HPV types 16, 18, 31 and 33. 10 out of these 55 cases were positive for HPV-16, 34 were positive for HPV-18, 4 were positive for HPV 31 and 5 were positive for HPV 33. 2 samples were found positive for both HPV-16 and HPV-18 (Table no. 2). Therefore, in the present study, HPV-18 infection was observed to be the most common (7.2%), followed by HPV-16 (2.4%), HPV-33 (1%) and HPV-31(0.8%).

The present study also showed high HPV prevalence in women with different age groups and was seen maximum upto 71.43% in women with > 65 years age group [Table no. 3(a)]. Women married before 18 years of age showed increased HPV prevalence (48.44%) [Table no. 3(b)]. HPV prevalence was also seen maximum in women with 4 or more children (50%) as compared to those who had less than 4 children [Table no. 3(c)].

The association between HPV infection and different demographic characters like age, age at marriage and parity was statistically calculated by using Chi square test of significance and the study showed significance association ($p<0.05$) of HPV infection with age, age at marriage and parity.

Discussion:

Study of the available literature shows the existence of more than 30 subtypes of genital HPV. However, the common carcinogenic strains have been reported to be HPV 16 and 18. Studies on the detection of high risk HPV

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strains using molecular genetics techniques have scarcely been carried out in the northern states of India.

Genital infection with HPV is one of the most common sexually transmitted disease. Its prevalence in young women ranges from 20 to 46 % in various countries (Evander et al., 1995). Cervical cancer develops from the progression of unclear HPV infection to high grade and eventually to invasive disease (Stanley, 2003). Specific types of HPV are associated with cervical cancer (Bosch et al., 1995) and there are approximately 100 fold chances of cervical cancer in women with normal cytology but infected with HR-HPV strain as compared to uninfected women (Rozendaal et al., 1996).

With the advent of molecular genetics techniques, particularly the PCR, it is possible to detect very low quantities of HPV-DNA and to subtype the commonly occurring HPV in cervical scrape smears.

In the state of Jammu and Kashmir, the study is first of its kind and has been carried out with the aim to detect the incidence of HPV infection in the suspected women and to find out the prevalence of HR-HPV types 16, 18, 31 and 33.

The prevalence of HPV using consensus primer among women with some genital or cervical complaints in the index study was 40.8% which was higher than that observed in previous studies performed using the same HPV testing protocols (Aggarwal et al., 2006). HR-HPV types 16, 18, 31 and 33 were detected in 11.4% of the entire samples and in 26.96% of the samples positive for HPV DNA. The available literature about the prevalence of HPV in women with abnormal cervical cytology varies from 20 to 40% in different countries (Table no. 4).

Previous data about the prevalence of HPV infection in general population of some of our neighbouring countries ranges from 8 to 50%. The burden of HPV infection in general population of Nepal was 8.6% (Sherpa et al., 2010) and ranges from 15-18% in three provinces of China (Dai et al., 2006; Li et al., 2006 and Wu et al., 2007).

In the high risk areas in Asia and Africa such as Mangolia, its prevalence was 35% (Dondog et al., 2008) and Guinea it was 51% (Keita et al., 2009).

Studies on the detection of HPV strains using molecular genetics techniques had been carried out in different parts of India. The available literature shows that the prevalence of HPV in women ranges from 8 to 40% in different parts of India (Table no. 5).

The prevalence of High risk HPV types in the present study was in accordance with that reported by Duttagupta et al. (2004) and Aggarwal et al. (2006).

There was found significant age related difference in the distribution of HPV in the index study. Duttagupta et al. (2004), Aggarwal et al. (2006) and Chaouki et al. (1998), Dutta et al. (2012) and Sarma et al. (2013) reported similar findings. Women who had 4 or more children were more prone to HPV infection as compared to those women with less children. The difference was found statistically significant. Aggarwal et al. (2006), Dutta et al. (2012) and Sarma et al. (2013) reported similar findings. Lazcano et al. (2001) and Duttagupta et al. (2004) didn't observe any significant association of HPV infection with parity.

Significant difference of HPV distribution with age at marriage (Age at first intercourse) was detected. Sarma et al. (2013) reported similar findings. This confirms that early marriages which results in longer sexual life in women and had chances of more pregnancies, results in developing more chances of cervical cancer in these females due to immature cervix and more wear and tear due to longer sexual life and more pregnancies. However, Duttagupta et al. (2004) and Aggarwal et al. (2006) didn't find any significant association of HPV with age at marriage.

In the index study, four types of HR-HPV types had been included as they were more prevalent in this part of the world especially HPV 16 and 18. We observed that these HR-HPV DNA were present in 11.4% of the entire sample. The figure reported was almost nearer to previous studies reported from India by Rozendaal et al. (1996), Gopalkrishna et al. (2000), Sarnath et al. (2002), Aggarwal et al. (2006) and Sarma et al. (2013).

Thus we find that cervical screening practices are inconsistent in India especially in the rural areas. Pap smear analysis is used as the only method for the screening of women in the hospitals which has limitations. Often the cytological interpretations becomes faulty if the smear is inflammatory, a situation not infrequent among women from low background.

Conclusion:

Thus we find that there is high prevalence of HPV infection in the women of Jammu region of Jammu and Kashmir and there is a great need of HPV DNA testing in cervical cancer screening and regular follow up of HPV positive cases. Also, there is great need for the screening of women from remote villages and rural areas of India as these parts are more prone to infection and are usually ignored because of lack of funds and infrastructure.

Acknowledgement:

Authors are thankful to Jammu and Kashmir State Council for Science and Technology, J&K Govt., Jammu and Kashmir State for providing financial assistance for present study.

TABLES

Table No. 1: Primer sequences used for the detection of different HPV genotypes.

Primer	Sequence	Product size (bp)	References
HPV consensus	F: CGG TCG GGA CCG AAA ACG G R: AGC ATG CGG TAT ACT GTC TC	240	Chatterjee et al., 2005
HPV 16	F: ATT AGT GAG TAT AGA CAT TA R: GGC TTT TGA CAG TTA ATA CA	109	Aggarwal et al., 2006
HPV 18	F: ACT ATG GCG CGC TTT GAG GAT CCA R GGT TTC TGG CAC CGC AGG CA	334	Aggarwal et al., 2006
HPV 31	F: TGA ACC GAA AAC GGT TGG TA R: CTC ATC TGA GCT GTC GGG TA	613	Carvalho et al., 2010
HPV 33	F: AGT AGG GTG TAA CCG AAA GC R: CTT GAG GAC ACA AAG GTC TT	411	Carvalho et al., 2010

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Table No. 2: Percent distribution of different HPV genotypes in women of Jammu region.

Samples	Number	Percentage (%)
HPV positive	204	40.8
HPV negative	296	59.2
HPV-16	10	2.0
HPV-18	34	6.8
HPV-31	4	0.8
HPV-33	5	1.0
HPV-16 & 18	2	0.4
Other HPV types	149	29.8

Table No. 3: Association of different demographic factors with HPV infection.

a) Association of Age factor with HPV infection.				
Age (yrs)	Number of women	HPV positive	HPV negative	HPV positive percentage
26-35	170	62	108	36.47
36-45	214	76	138	35.51
46-55	68	38	30	55.88
56-65	34	18	16	52.94
>65	14	10	4	71.43
b) Association of Age at marriage with HPV infection.				
Age at marriage (yrs)	Number of women	HPV positive	HPV negative	HPV positive percentage
<18	128	62	66	48.44
18-24	338	136	202	40.24
25-28	28	6	22	21.43
>28	6	0	6	0
c) Association of parity with HPV infection.				
Parity	Number of women	HPV positive	HPV negative	HPV positive percentage
0	12	4	8	33.33
1	28	8	20	28.57
2	266	58	208	21.8
3	146	60	86	41.1
≥4	148	74	74	50

Table No. 4: Prevalence of HPV in women with abnormal cervical cytology in different countries

Author	Place	Women enrolled (Number)	Positivity	Method
Chaouki et al. (1998)	Morocco	185	20.5%	PCR
Womack et al (2000)	Zimbabwe	556	39%	Hybrid capture
Rolon et al (2000)	Paraguay	101	20% (HPV) 5.5% (HPV 16)	PCR
Ekalaksananan et al (2001)	Thailand	174	21% (Type 6 and 11)	PCR
Thomas et al (2004)	Nigeria	932	24.8%	PCR and southern blotting
Dai et al (2006)	China, Shanxi province	662	14.8% (HPV)	PCR
Li et al (2006)	China, Shenyang city	685	16.8% (HPV)	PCR
Wu et al (2007)	China, Shenzhen city	534	18.4%	PCR
Rama et al (2008)	Brazil	2300	17.8%(HR-HPV)	PCR
Keita et al (2009)	Guinea	831	50.8%	PCR
Sherpa et al (2010)	Nepal	932	8.6% (HPV)	pcr

Table No. 5: Prevalence of HPV in women in different parts of India.

Authors	Place	Subjects enrolled	Number	Positivity	
Gopalkrishna et al. (2000)	New Delhi	Women with sexually transmitted diseases and cervical precancerous and cancerous lesions	30	13.3% (HPV 16)	PCR
Sarnarth et al. (2002)	Mumbai	Women attending radiotherapy department of Tata Memorial Hospital with clinically normal cervix	164	15.2% (HPV 16/18)	PCR and Southern blotting
Franceschi et al. (2003)	Chennai	Admitted patients and their visitors with risk of cervical cancer from Chennai	184	27.7% (HPV DNA) 16.32% (HPV 16 and 18)	PCR and Southern blotting
Duttagupta et al. (2004)	West Bengal	Rural women from eastern India with low socioeconomic families	792	8.8%	PCR
Clifford et al. (2005)	Tamil Nadu	Women randomly selected from general population	1179	14.2% (HPV DNA)	PCR based EIA
Aggarwal et al. (2006)	Chandigarh	Hospital based study of women with benign cervical cytology	472	36.8% (HPV DNA) 8.2% (HPV 16 & 18)	PCR
Dutta et al. (2012)	Eastern India	HPV prevalence in women of Eastern India without cervical cancer	2501	9.9% (HPV DNA), 2% (HPV 16 & 18)	PCR
Srivastava et al. (2012)	Uttar Pradesh	A population based study of asymptomatic women of eastern Uttar pradesh	2424	9.9% (HPV DNA)	PCR
Sarma et al. (2013)	Guwahati	Demographic characteristic of HPV infection in women-a hospital based study	226	9.73% (HPV DNA)	PCR
Kaur et al. (2014)	Amritsar	Prevalence of HPV infection in patients with cervical lesions	100	10% (HPV DNA), 6% (HPV 18) and 2% (HPV 16)	PCR
Present study	Jammu and Kashmir	HPV prevalence among women screened for cervical cancer	500	40.8% (HPV DNA),11% (HR HPV 16,18,31 &33)	PCR

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FIGURES

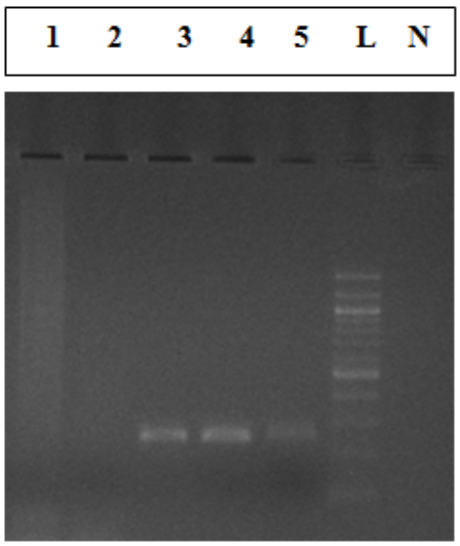

Fig. 1: Gel image of PCR product of HPV consensus primer showing band size of 240bp in lane no. 3, 4 and 5

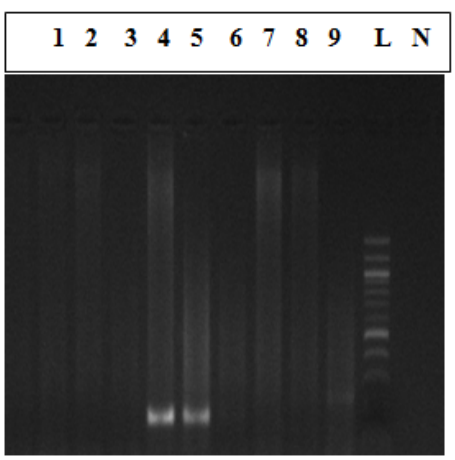

Fig. 2: Gel image of PCR product of HPV 16 primer showing band size of 109bp in lane no. 4 and 5

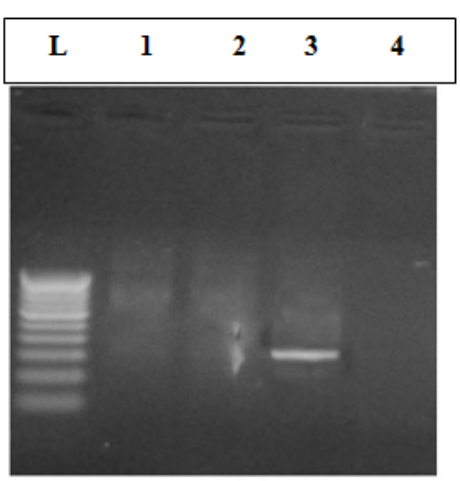

Fig. 3: Gel image of PCR product of HPV 18 primer showing band size of 334bp in lane no.3

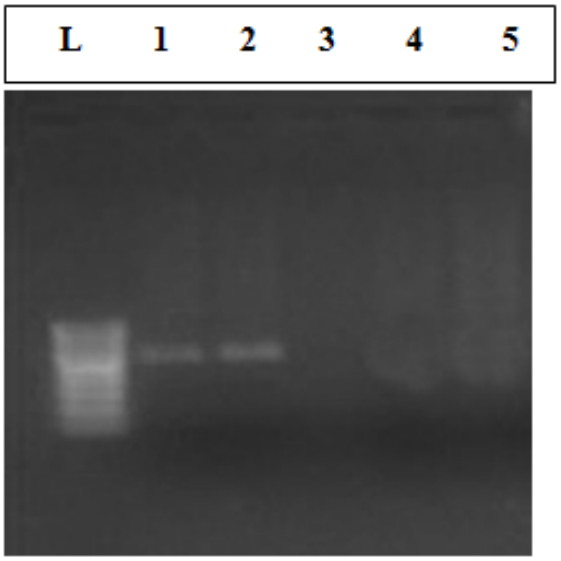

Fig. 4: Gel image of PCR product of HPV 31 primer showing band size of 613bp in lane no.1 and 2.

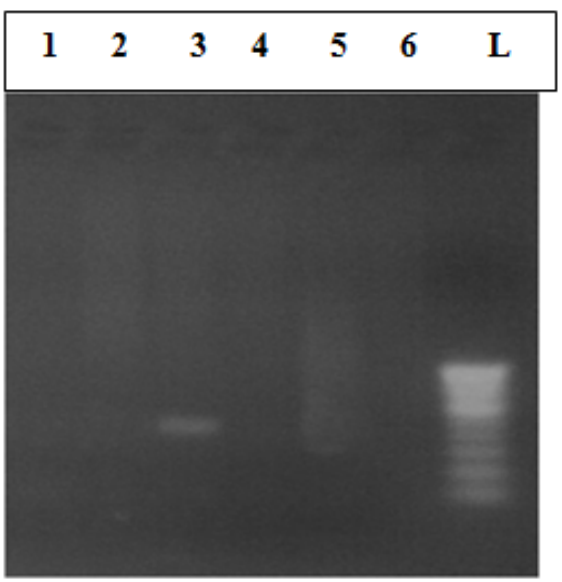

Fig. 5: Gel image of PCR product of HPV 33 primer showing band size of 411bp in lane no.3

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