

RESEARCH ARTICLE

Detection of Genital HPV Infection Using Urine Samples: a Population Based Study in India

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Abstract

Background: Cervical cancer is the second commonest cancer among Indian women and its association with human papilloma virus (HPV) is well established. This preventable cancer accounts for the maximum number of cancer related deaths among rural Indian women. Unlike in developed countries there are no organized cervical cancer screening programmes in India due to lack of resources and manpower. **Objective:** To detect genital HPV infection using urine samples among asymptomatic rural women in the age group of 18-65 years. **Materials and Methods:** The study area chosen was Perdoor village in Udupi Taluk, Karnataka State and all the women in the age group of 18-65 years formed the study cohort. A cross sectional study was conducted by house visits and 1,305 women were enrolled in the study. After taking written informed consent a data sheet was filled and early stream random urine samples were collected, transported to a laboratory at 4°C and aliquoted. Samples were tested using nested HPV PCR with PGMY09/11 and GP5+/6+ primers. Positive cases were genotyped by sequence analysis. **Results:** Study participants included 1,134 sexually active and 171 unmarried women with a mean age at marriage of 22.1 (SD=3.9) years. Study area showed high female literacy rate of 86.6%. Five urine samples tested positive for HPV DNA (0.4%). **Conclusions:** We found very low genital HPV infection rate among women from monogamous community. This is the first major population based study carried out among asymptomatic rural women to detect genital HPV infection from Karnataka using urine samples.

Keywords: Human papillomavirus - urine sample - asymptomatic women - Karnataka - nested PCR

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Introduction

Eventhough cervical cancer is showing a declining trend in India it continues to be a major cause of cancer related mortality among Indian women. Worldwide cervical cancer is the fourth commonest cancer among women and the second commonest cancer occurring in Indian women. Association of Human Papillomavirus (HPV) with cervical cancer is well proven. Epidemiological studies on cervical cancer cases from India have found HPV positivity as high as 98% (Das, 1992) (Bhatla et al., 2008). Persistent infection with high risk human papillomavirus is the most important risk factor for developing cervical cancer (Walboomers et al., 1999).

Cervical cancer is preventable by regular screening, vaccination, early detection and management of precancerous lesions. For decades Pap smear screening has played a vital role in cervical cancer prevention in the developed worlds. But this requires sophisticated health

care infrastructure, trained cytopathologists, and multiple hospital visits which become an uphill task for women from developing nations. Unlike in developed countries there are no organized cervical cancer screening programmes in India due to lack of resources and manpower. Lack of awareness among healthy rural women regarding regular screening is another major hurdle in implementing such programmes. Last two decades witnessed the development of HPV molecular testing using cervical, vaginal and urine samples and US-FDA has approved HPV testing for cancer screening in 2014 which requires expertise and resources.

About 70% Indians live in villages and huge gap exists in the field of education, basic amenities and health care facilities between urban and rural areas. Even cancer incidence data published by major cancer registries is mainly collected from urban areas and medical colleges. Among healthy population HPV prevalence of 7-13% reported according to Indian studies (Bruni et al., 2010). There is scarcity of data from rural India and Northeast

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region and information about the epidemiologic data from every subpopulation is required prior any intervention like vaccine implementation (Nigam et al., 2014). In this context we decided to carry out a community study to detect genital HPV infection rate among rural women using urine samples.

Materials and Methods

Study design

A cross sectional study was carried out among asymptomatic rural women in the age group of 18-65 years of age during the period August 2013-May 2015 in a village in Udupi taluk with a population of 10,565 (Sabeena et al., 2015). Permission from Directorate of health and Family welfare services, Government of Karnataka was taken and was approved by Institutional Ethical Committee of Kasturba Medical College, Manipal University, and Manipal (IEC244/2013) before initiating the study. Collaboration with the local Primary Health care Centre and subcentre was established to reach the participants.

Sample size and Sampling

Minimum sample size of 704 married women was calculated with the assumption that the expected prevalence of HPV among married women as 12% at 95% confidence interval and 20% relative precision (Senapathy et al., 2011).

Inclusion criteria: Physically and mentally competent women in the age group of 18-65 who were permanent residents of Perdoor grampanchayath formed the study population and women willing to participate in the study were enrolled by house visits. Even unmarried women, pregnant women and women who had undergone hysterectomy for reasons other than cervical cancer were included.

Subject recruitment

The objective and public health importance of the study was explained to local government doctor, staff at Primary Health Centre and subcentre and to ASHA (Accredited Social Health Activists) workers. Households were visited with ASHA workers if at least one lady in the age group of 18-65 years was residing in that house. Before enrollment into the study all participants were given subject information sheet in local language (Kannada). Women who agreed to participate were asked to sign the written informed consent. Epidemiological data on age, occupation, literacy, socioeconomic status, diet, wood smoke exposure, tobacco exposure, alcohol consumption, marital history, reproductive aspects and genital hygiene were obtained from all enrolled women.

Sample collection

An early stream random urine sample was collected and transported to laboratory at 4-8°C.

Sample aliquoting:

About 20-25ml urine sample collected per participant

was mixed well and centrifuged at 3800g for 20 minutes at 4°C in a refrigerated centrifuge (Sorvall Legend XTR Thermo Fisher Scientific, Germany). The middle opaque phase was collected, transferred into 1.5ml microcentrifuge tube and centrifuged at 16,000g for 15minutes at 4°C. The pellets obtained from this were suspended in PBS, aliquoted and stored at -70--80 °C (Tanzi et al., 2013). DNA was extracted using Qiagen viral DNA kit as per manufactures instructions.

PCR inhibition by urine samples

To check whether the urine sample is inhibiting PCR reaction, urine sample collected from a person not from the study area was spiked with a sample positive for HPV. Viral DNA was extracted from spiked sample and tested for HPV by standardized protocol. The sample was tested positive for HPV and the inhibition of PCR by urine was ruled out. This experiment validates the use of urine for detection of HPV in the study.

HPV DNA testing

Samples were tested for Human Papillomavirus (HPV) by nested PCR using PGMY09/11 and GP5+/6+ primer sets (R., 1995; Gravitt et al., 2000). Genotyping of positive cases was done by sequence analysis. Pap smear and cervical samples were taken from all consenting married women with HPV infection.

Data analysis

Descriptive statistics were reported using mean \pm SD for age and frequency and percentage for categorical variables. Statistical analysis was done using Epi Info7.

Results

From the study area 1324 women were approached by house visits of which 1307 agreed to participate in the study with a response rate of 98.7%. All these women were personally interviewed and early stream random urine samples were collected. Two samples were lost during transportation and 1305 samples were aliquoted and tested by HPV nested PCR. Among the five urine samples tested positive for HPV DNA one sample could be typed as HPV-18. Only five urine samples were tested positive for HPV (0.4%). As shown in Table-1, around half of the participants enrolled in the study were in the age group of

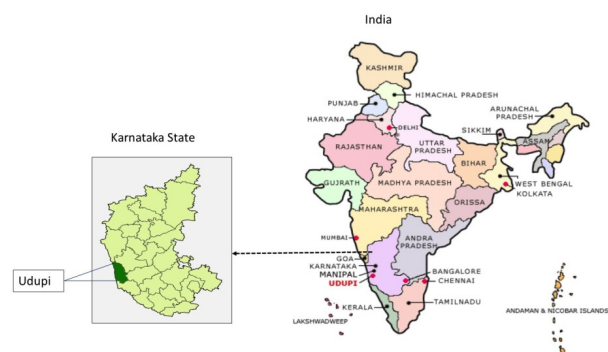
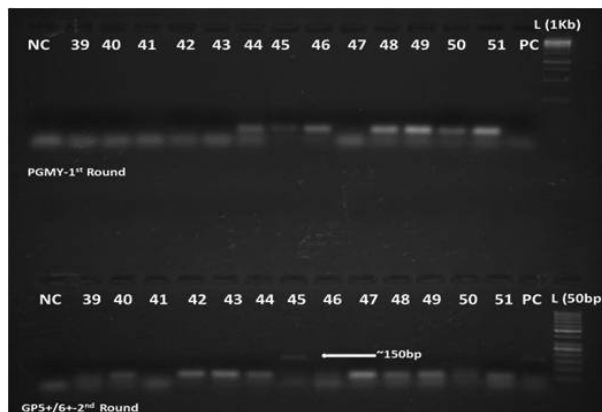


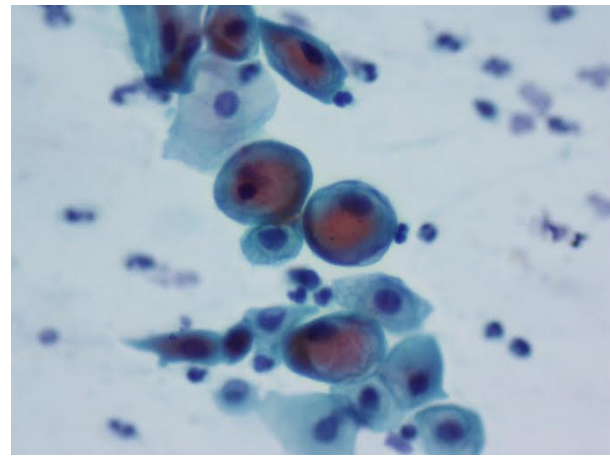
Figure 1. Geographical map showing study area in Udupi district, Karnataka, India

Table 1. Sociodemographic characteristics of HPV study participants (n=1305)

Sociodemographic characteristics	Number (%)	HPV DNA+ve in urine samples
Age groups(years)		
18-24	31 (12.4)	0
25-34	323 (24.8)	1
35-44	355 (27.2)	2
45-54	307 (23.5)	1
55-60	198 (15.2)	1
61-65	91 (6.9)	0
Religion		
Hindu	1218 (93.3)	5
Muslim	85 (6.5)	0
Christian	2 (0.2)	0
Socioeconomic status		
Low	805 (61.7)	4
Middle	489 (37.5)	1
High	11 (0.8)	0
Literacy		
Illiterate	183 (14.0)	3
Primary	210 (16.1)	0
Middle school	364 (27.9)	1
High school	326 (25.0)	0
PUC	117 (9.0)	0
Graduate	105 (8.1)	1
Occupation		
Home maker	786 (60.2)	2
Unskilled	379 (29.0)	0
Semiskilled	12 (1.0)	0
Skilled	94 (7.3)	3
Students	34 (2.6)	0
Age at marriage (1134)		
≤15	25(2.2)	0
16-17	58(5.1)	0
18-19	187(16.5)	2
≥20	864(76.2)	3

**Figure 2. Gel Electrophoresis Picture Showing Nested HPV PCR Products**

25-44 years (52.8%). Mean age of the study participants was 38.8 years (SD=12.3) and mean age at marriage was found to be 22.1 years (SD=3.9). All the women detected to be infected with HPV were above 30 years of age including one 60 year old postmenopausal lady. Among the study population majority were Hindus (93.3%) and 0.2% were Christians. According to modified Udai

**Figure 3. Cytology showing atypical squamous cells with nucleomegaly and perinuclear halo suggestive of HPV infection PAPx400**

Pareek scale 805 women were from low socioeconomic status (score<40) and 489 women were from middle socioeconomic status (score 40-70). A high literacy rate of 86.6 % was noted among the study participants and most of them had received education at least up to middle class. Two-third of the women who participated in the study were home makers (60.3%). Cashew factory workers constituted the major portion of working women and beedi rolling was done by 10.9 % women at home.

Most of the families lived in mixed houses built of stones, cement and tiled roof (80.7%). Eventhough almost all households had direct access to drinking water facility, running water facility inside house was reported by only 596 women (45.7%) and this is often related to genital hygiene. After sexual contact 257 women (19.7%) reported washing of genital areas. Majority of the study population were non-vegetarians (94.3%) and more than 80% households used firewood for cooking. Age at marriage was 20 or above for three-fourth of the enrolled women. Only 24 years women had undergone Pap smear testing (1.8%). Contact with multiple sexual partners was reported by a widow.

Women detected to be infected with HPV were called to village subcentre on a prefixed date. Pap smear and cervical samples were taken from four women after taking consent and all cervical samples were tested positive for HPV. Among the Pap smears collected three were normal smears. The fourth one collected from a widow with multiple partners was suggestive of Atypical Squamous Cells of Undermined Significance (ASC-US) as shown in Figure-3 and the cervical swab collected from the same participant was typed as HPV-18.

Discussion

Very low detection rate of genital HPV infection among rural women in the current study may be explained by the higher age at initiation of sexual activity and widely practiced monogamous sexual life style among both men and women. Mean age at marriage for Indian women is 21.6 years as per 2011 census, but half of the rural women and one third of the urban women in India are married

between 18-20 years of age. In Perdoor study two thirds of the study participants got married at or after 20 years of age. Data from a community based screening trial from Osmanabad district, Maharashtra, India conducted among women in the age group of 30-59 years reported a HPV prevalence of 10.3%. But the mean age at first sexual contact was 15 years and high number of teenage pregnancies were reported among these women (Sauvaet et al., 2011). Immature transformation zone is mainly susceptible to HPV infection and younger age at sexual debut is a well-recognized risk factor for HPV infection (Veldhuijzen et al., 2010). In Perdoor study only one woman had multiple sexual partners and two women reported extramarital relations of their husbands. Almost all participants had single life time sexual partner. Multiple sexual partners increase the risk of incident as well as persistent HPV infections (Mollers et al., 2013). Women with 4 or more sexual partners were almost four times more at risk of having any HPV infection compared to women with single partner (Nahar et al., 2014).

The causal association of HPV with cervical cancer is well accepted and the incidence of cervical cancer cases can be related with HPV prevalence in that area. Cervical cancer is the second commonest cancer among women from this area and like most parts of India number of cervical cancers are coming down. There are no population data regarding asymptomatic genital HPV infection from this part of country. Recently published hospital based study from Udupi district found lower HPV prevalence of 82.5% among cervical cancer samples in contrast to previous Indian studies (Kabekkodu et al., 2015). Two recent studies from Central South China and Iran also reported HPV prevalence of 75.7% and 76% in cervical cancers respectively (Yang et al., 2014) (Jalilvand et al., 2015). A population based study conducted in Southern Iran revealed very low genital HPV prevalence of 0.6% and clinic based study from the same locality reported prevalence of 5% among women. Among 799 women in the age group of 21-50 years participated in the Iran study cervical samples from only 5 pregnant women were tested positive (Eghbali et al., 2012).

HPV detection rate observed in our study is lower than what observed in previous Indian studies. Population studies from India report varying prevalence rates of 16.9% among women from Tamilnadu to 3.6% from Andaman Nicobar (Franceschi et al., 2005; Parvez et al., 2012; Sureshkumar et al., 2015). A study conducted using urine samples among tribal women in the age group of 9-25 years from India found a higher HPV prevalence of 12.9% and more than two third of the participants were infected with High risk HPV (Sharma et al., 2015). Important factors to be considered is the age of study participants, younger age at sexual debut, widely practiced polygamy, home deliveries and multiparity among these indigenous subgroups and this data cannot be representative of entire India.

In the major population study from Dindigal, Tamilnadu, India home visits were paid to inform the rural women about the study and were invited to the clinic for vaginal examination (Franceschi et al., 2005). Higher refusal rate was reported in this study. Other two major

population studies enrolled women from Gynecology clinics and cervical cancer screening programmes (Srivastava et al., 2012; Sureshkumar et al., 2015). Globally higher prevalence is seen in cancer screening clinics as most of the women might be having some gynecological problems and willingness for participation depends on their awareness and health seeking behavior. Women participating in voluntary cervical cancer screening programmes showed higher prevalence compared to population based survey conducted in Italy (Giorgi Rossi et al., 2010). Clinic based reporting of prevalence data should be adjusted downward according to population based surveys (Gouws et al., 2008). In our study asymptomatic women were included by house to house visits and urine samples were collected at home and transported to laboratory.

In developing countries cervical cancer is always associated with poverty, illiteracy and multiparity. These studies show that with literacy, better adoption of family planning practices and genital hygiene a reduction in HPV infection is achievable. Perdoor village in Udupi district shows a higher female literacy rate and better standards of living compared to the rest of India (Nair et al., 2000). Prevalence study carried out in North India found higher prevalence among rural women following non vegetarian diet and using homemade pads during menstruation (Srivastava et al., 2012). In our study 95% were following non vegetarian diet and three fourth of the study population used homemade pads.

Last two decades witnessed HPV detection studies using urine samples and it shows a sensitivity of 90% and specificity of 70% in detecting genital infection among women (O'Leary et al., 2011). Urine is an adequate alternative specimen to monitor HPV prevalence among women when there are cultural and religious hardships in obtaining genital samples (Vorsters et al., 2012) (Enerly et al., 2013) (Mendez et al., 2014). Eventhough urine samples are less sensitive compared to cervical samples, it is inexpensive and more socially acceptable for large epidemiological surveys in developing countries. Urine samples are moderately sensitive for detecting HPV and highly specific for detecting any HPV including HPV16 and 18 (Pathak et al., 2014). Urine based screening can reduce the number of false positive results and unnecessary invasive procedures. HPV detection in urine can be improved by using the first void urine (Vorsters et al., 2014). In our study women who had undergone hysterectomy for benign reasons were also included as there are case reports of HPV infection of vagina among hysterectomised women (D'Souza G, 2012). To the best of our knowledge this is the first population study conducted among asymptomatic women from Karnataka regarding genital HPV infection using urine samples. Another study conducted among the general population from North Karnataka using salivary samples found alarmingly high prevalence of HPV in the order of 64.3% and HPV-18 was the predominant type (Kulkarni et al., 2011). Study claimed to collect 369 salivary samples irrespective of sex and age of participants from rural and urban areas. When prevalence from a population group is discussed age, sex and immunity matters as HPV shows higher prevalence

among adolescents and immunocompromised individuals.

All the women tested HPV-positive in Perdoor study were above 30 years of age including one 65 year old postmenopausal lady. Population studies from various parts of India found increased HPV prevalence as age advances (Sankaranarayanan et al., 2005; Asiaf et al., 2012; Srivastava et al., 2012). Highest HPV prevalence occurs soon after initiation of sexual activity among young women in the age group of 18-24 years and gradually comes down with a second peak after the fifth decade of life (Trottier et al., 2010). In this context WHO recommendation of screening of women 35 years or above and every five years for three tests in life time will be a more cost effective approach in resource poor settings (Aggarwal, 2014). In our study male condom usage was reported by few couples for spacing of pregnancies and male condoms protect from HPV infections partially (Moscicki et al., 2012). Smokeless tobacco use was found among few women in the form of chewing pan or nasal powder. Very few women were found to be taking alcoholic beverages. Tobacco and alcohol increase the risk of persistent High risk HPV infection and unsafe sexual practices (Veldhuijzen et al., 2010). Male circumcision rate in our study was 6.4% and according to meta-analysis study circumcision reduces HPV infection and high risk HPV prevalence among men (Albero et al., 2012). All the cervical samples collected from Perdoor women were HPV DNA positive showing 100% concordance. Another study by Prusty et al also found 100% agreement in HPV positivity between cervical and urine samples (Prusty et al., 2005). A meta-analysis study comparing the sensitivity and specificity of Pap smear and HPV DNA found better sensitivity and specificity of HPV-DNA testing in the order of 94.6% and 94.2% (Koliopoulos et al., 2007). Cervical cancer screening rate is very low among women from Karnataka and from this study area very few women had undergone Pap smear testing. Another factor to be considered is the fall in the incidence of both HPV infections and cervical cancer among women from the study area.

Strength of the study: This is the first major population study conducted among rural women from Karnataka to detect genital HPV infection using urine samples. We could screen 1305 rural women by house visits for genital HPV infection from an area from where, there was no previous community based study in this aspect using genitourinary samples. Modified aliquoting of urine samples was employed for better DNA extraction.

Limitation: Main limitation of our study was the non-probability convenient sampling.

Research implications: Urine sampling enabled wider coverage of rural women including unmarried and pregnant women for whom genital sampling would have been a difficult option in the current study. This sampling strategy will be more acceptable to rural women with low screening compliance very low genital HPV prevalence was observed among rural asymptomatic women in the present study. studies.

In conclusion, Studies concerning HPV prevalence is required from different parts of India as there is vast social, linguistic and religious diversity across the country. Comprehensive data on prevalence of specific genotypes

from various parts will be helpful in choosing appropriate prophylactic vaccines.

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