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Original Article

Oncogenic human papillomavirus DNA in female sex workers of Bihar, India

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ABSTRACT

Objective: Human papillomavirus (HPV) is a sexually transmitted virus which play a vital role to developing Cervical cancer. It is presumed and accepted that sexually exposed women will have occurred HPV infection at least once in their whole life. Hence, determined the actual cause of transmission of HPV infection in sexually active women with single sex partner that is married women and sexually active women with multiple sex partners that is female sex works (FSWs).

Methods: A total 197 urine sample of FSWs and MW has been utilized through RT-PCR technique for HR-HPV detection. DNA extracted from urine samples and estimation and purification of DNA, used RT-PCR technique for HPV-16 and HPV-18 detection.

Results: The overall prevalence of HR-HPV was detected to be 16.75% (33/197) and 83.25% (164 out 197) negative results reported in both studied subjects. The results analysis of HPV-16 and HPV-18 reveals in which prevalence of HPV-16 was 14.21% (28/197) and HPV-18 was detected 2.53% (5/197) in studied subjects in both study groups. The prevalence of HPV-16 and HPV-18 among MW was high (18.75%) and HPV-16 and HPV-18 was low (14.85%) in FSWs. It did not differ significantly of HR-HPV prevalence in MW who has single sex partner and FSWs who has multiple sex partners.

Conclusion: The study indicates that oncogenic HPV prevalence did not different significantly in multiple sex partners, that is, FSWs and single sex partner, that is, MW. The probability of genital hygiene rather than multiple sexual partners stands more apt as a cause of HPV infection. This study advises to develop more awareness program about genital hygiene in women to reduce the HPV infection and can be prevented from cervical cancer. Hence, genital hygiene may be reducing the burden of HPV infection in women.

Keywords: Human papillomavirus, female sex workers, sexually transmitted disease, cervix cancer

Introduction

Cervix uteri is the 2nd utmost frequently diagnosed cancer among women in India.[1] Human papillomavirus (HPV) is sexually transmitted disease (STD) which paly a prime role to developing cervical cancer. Cervical cancer is more common in developing country and closely related to many risk factors such as as multiple sex partners, multiple pregnancy, refractory reproduction tract infection, lifestyle, early age of marriage, poor genital hygiene, early age of intercourse, risky sexual behavior, and many more. About 80% sexually exposed women infected due to persistent of HPV virus leads to invasive uterine cervix cancer.[2] HPV associated with uterine cervix cancer has been well established, with HPV being documented as the cause of most of the cases of cervix carcinoma and cervical epithelial cell dysplasia.[3,4] Persistent of HPV infection is primary causative agent of Cervical Intraepithelial Neoplasia (CIN) in women population. Of more than 100 HPV genotype strain classified into high risk and low risk type have been identified. HPV mainly comes under two genotype high risk genotype strains: HPV-16 and HPV-18 and low risk genotype strain: HPV-6 and HPV-11. High risk HPV-16 and HPV-18 is transmitted sexually and when in contact with transformation zone of cervix is known to contribute to invasive cervical cancer (ICC).[5] Some studies demonstrated to number of sex partners as one of the most important risk factors associated with the presence of genital HPV DNA. The prevalence of HPV infection peak among younger women, but it is not clear whether younger women are more exposed to HPV infection than older women or whether clearance is delayed.[6-8] Female

sex workers (FSWs), that is, multiple sexual partners increase the risk of incidence as well as persistent HPV infection.[9] Women with four or more sexual partners were almost increase 4 times more at risk of having any HPV infection compared to women with single partner.[10] Without protected sex with multiple number of sex partners carries high risk acquiring HR-HPV in women.

Oncogenic HPV is essential to growth of cervix cancer with certain duration period for persistent infection. Various sexually exposed individual women become HPV infection after beginning their earliest sexual connection.[11] Even those that persistent of HPV infection take few months is generally cleared naturally, HPV infection is typically transient. Progression of uteri cervix carcinoma and CIN is almost constantly preceded by a persistent HR-HPV infection. This is abnormal changes in tissue of the cervix (dysplasia) and diagnosed from histological analysis of cervical dysplasia. On the basis of abnormality level, it is classified into three grades: CIN Grade-1 (CIN1), that is, Mild dysplasia and genital warts, CIN Grade-2 (CIN2), that is, Moderate dysplasia and CIN Grade-3 (CIN3), that is, Severe dysplasia. CIN1 is less serious than CIN3, CIN2/CIN3 are pre-cancers that can develop into ICC. Persistent infection with HPV-16 and HPV-18 is recognized to induce cervix uteri carcinoma, mainly in the occurrence of cofactors.[5] Infection of HPV is very common in adolescent or young female of sexual exposure in their first decade. Persistence of HPV infection and pre-cancer is well recognized within 5–10 years from <10% new infection.[5,12] It seems that FSWs, because of their early age of sexual intercourse, repetitive sexual exposure to without protected sex may be the victims of HPV infection as well as act as accumulation of HR-HPV transmission in the society, especially in India.[5] FSWs have multiple sex partners are associated with high risk exposure of HPV infection in women.[13,14] At less than 20-year of FSWs was risk of acquiring human immunodeficiency virus (HIV) as compared with old age FSWs.[15] It is associated with HR-HPV in FSWs leading to cervix uteri cancer is further depolarized by the presence of coinfection of HIV.[16]

This may be led to an important contribution to progression of cervical cancer to India's nationwide burden, as there are estimated 2,900,000 FSWs functional in India[17] and large number of them HIV infected. Hence, this study was considered obligatory to analyze the magnitude of problematic of HR-HPV among FSWs in Bihar, India and aim to focus on actual mode of transmission of HPV infection and sexual activities, personal hygiene, age group, and risk factor among FSWs and married women (MW) in healthy subjects.

Materials and Methods

Study population and sample size

The study was done at Mahavir Cancer Sansthan and Research Center Patna, India. Healthy FSWs, that is, multiple sex workers and sexually exposed women, that is, single sex partner participated in this study and number of sample size was 197 urine specimen, age range between 17 and 52 years.

Inclusion and exclusion criteria

All women who met inclusion criteria were invited to participant. Some inclusion and exclusion criteria of this study as below:

Inclusion criteria

Women: (i) Who were - healthy/disease free women, (ii) who were - having single sex partner, that is, MW, (iii) who were - have multiple sex partner, that is, FSWs, (iv) who were - not HPV vaccinated women, (v) age range between 17 and 52 years, (vi) who were - willing to participate in this study and were ready to fill consent form and questionnaire, and (vii) Bihar region of India only.

Exclusion criteria

Women: (i) Who were - not sexually exposed women, that is, unmarried women, (ii) who were adolescent girls, (iii) who were - age below 16 years (iv) who were - above 52 years, (v) who were - pregnant women, (vi) who were - with any uterine cervical abnormalities and conformed with uterine carcinoma cases, (vii) who were - previous surgical procedure on the uteri cervix or hysterectomy, (viii) who were - menstrual cycle period during sampling, (ix) who were - screening of pap smear, liquid based cytology, etc., (x) who were - male sex partner of participant women, (xi) those who were - not willing to fill consent form and questionnaire of participate in the study, and (xii) those who were - out of Bihar state region of women.

Biological specimen collection

We have collected the 60mL urine samples in sterile collection container from healthy FSWs and MW. Further we have been preceded for DNA extraction.

DNA isolation

First, transfer the urine specimen from urine container to 20 mL conical tube. Centrifuged at 4000 RPM at 4°C temp. for 15–20 min and discarded the supernatant and keep pallet. First step repeated 3 times and taken total volume 60 mL urine specimen for adequate quantity of DNA. Added 200 mL sterile phosphate buffer solution in pallet of micro-centrifuge tube and vertex for 01 min for dissolving and mixing the pallet. Again, centrifuged the pallet tissue at 4000 RPM for 10 min. Previous step has repeated once again. Finally, find the tissue pallet from the urine sample and further preceded for DNA Extraction, we have used MyLab Lifesolutions manufacturer kit for DNA isolation. 200 µL sample taken in 2 mL micro-centrifuge tube, then added 20 µL Lysis Enhancer Buffer to the specimen and mixed well by vortex for 10 s and added 20 µL RNAout solution to specimen mixed well by vortex, incubated

at room temp. for 2 min. Added 200 µL Lysis Buffer-2 and mixed well by vortex to obtain a homologous solution. Incubated at 55°C temp. on dry bath machine for 10 min to promote protein digestion. Added 200 µL 98–100% ethanol to lysate. Mixed well by vortex for 5 s to yield a homologous solution and proceeded for DNA binding process.

Binding and washing of DNA

Added prepared lysate approx. 640 µL in spin membrane column to centrifuged for 1 min at 10,000× *g* RPM at room temp. Collection tube discarded and placed the spin membrane column into clean collection tube supplied by manufacturer. Proceeded for washing of DNA. Added 480 µL Wash Buffer 1 to spin column at center. Centrifuge spin column at 13,000× *g* for 3 min at room temp. Discarded the solution from collection tube. Given an empty spin by centrifuging at 13,000× *g* for 1 min a room temp. and discarded the collection tube. Placed the spin column in a sterile 1.5 mL micro centrifuged tube and proceeded to Elution buffer of DNA.

Elution buffer of DNA

Added 50 µL Elution Buffer to membrane containing spin column, incubated for 1 min at room temp. Centrifuged the spin column at 15,000× *g* for 1 min at room temp. The micro-centrifuge tube contains purified genome DNA and discarded spin column from micro-centrifuge tube, stored the eluted DNA at –20°C until use for further process.

Estimation and purity of DNA

We have used the QuantiFluor® dsDNA flurometer used for estimation of DNA. It comprises a fluorescent DNA-binding dye that allows sensitive quantitation of small amount of double stranded DNA in purified sample. The test is highly selective for dsDNA over other nucleic acid and is linear range of 0.05–200 ng of dsDNA input. The dye-based system provides concentrated QuantiFluor® dsDNA dye, dilution buffer and DNA standard. Reagent stored at 2–10°C and protected from sunlight. QuantiFluor® ds DNA system contains ×20 TE Buffer solution (pH 7.5), dsDNA Dye and DNA standard reagent**.** We prepared ×1 TE Buffer solution to added 19 mL Nuclease free water and mixed well and protected from sunlight. Prepared working solution to dilute the QuantiFluor® ds DNA dye 1:400 in ×1 TE buffer. Prepared Blank solution to added 200 µL of QuantiFluor® dsDNA Dye to an empty 0.5 mL PCR tube and protected from sunlight. Prepared working solution distributed 200 µl each 0.5 mL PCR tube and add 1µl sample to incubated prepared sample at room temp. for 05 min, protected from light. First calibrated screen the Quantus™ Fluorometer for standard solution and saved and put the Blank solution in Flurometer and calibrated and saved. Measured the DNA isolated specimen using Fluorometer. The number displayed represented the concentration of the DNA specimen. In this study, urine specimen ranged from 10ng/mL to 85ng/mL concentration of DNA purity.

Prepared master mixed for real time-PCR

Pathodetect™ HPV RT-PCR protocol is designed for *in vitro* detection of HR-HPV DNA from isolated DNA specimen. MyLab Lifesolutions kit for RT-PCR based on Taqman fluorogenic probe chemistry that uses 5' end nuclease activity of Taq DNA polymerase and enable the detection of precise PCR product as it accumulates during RT-PCR cycles. Following reagent provided by MyLab Lifesolutions manufacturer for master mix:

- Nuclease Free Water solution
- PCR Mix solution
- IC Detection Mix solution
- HPV-16 Detection Mix solution
- HPV-18 Mix solution.

Kept all reagents on icepack during experiment setup (as per manufacturer instruction). Taken and thaw gently all the components thoroughly and before using it mixed gently, spin down the content for 5 s and then test it immediately. Test Controls: HPV-16 and HPV-18 positive control. Used Nuclease free water as HPV negative control (-NC). Determined the number of reaction (N) to perform for each experiment. It is necessary to make excess reaction cocktail to allow for the control reaction and pipetting error. Prepared two master mix in 2.0 mL micro centrifuge tube for RT-PCR. One is for HPV-16 and another for HPV-18. Prepared 17.0 µL each volume of master mixed for genotype HPV-16 and HPV-18 in PCR tube to added specimen number of reactions to be set up for each mix reaction reagent mixed well by pipetting up and down gently but need not vertex. Centrifuged for few seconds to collect consents at bottom of the tube, and place the tube on icepack. Set up reaction strip tubes or plates and dispense 17 µL of above cocktail into each well as per the plate set up. Before moving the plate to the DNA handling area, pipette 8 µL of sample DNA/positive control/negative control into respective well as per the set up to make 25 µL of total volume. Cap the wells or sealed the plate with optical sealer. Centrifuged the plate for few seconds. Make sure that bubbles are eliminated from the bottom of the reaction tubes. Now setup plate experiment for RT-PCR and run the RT-PCR machine. Each Specimen put on the wells of RT-PCR and assigned target of genotype HPV-16, HPV-18, and internal control each other, respectively.

The assay is based on RT-PCR for the amplification of specific conserved target sequence of E6/E7 region of HPV-16 and HPV-18 and detection of target specific probe. β-globin gene is also detected as housekeeping gene to check for extraction efficiency and PCR inhibition. The test principle is based on Taqman technology which allows higher specificity and sensitivity. This kit uses one steps real time PCR with Taqman fluorogenic probes chemistry that uses the 5′nuclease activity of Taq DNA polymerase enzyme and enables the detection of a specific PCR product as its accumulation during PCR cycles. We have used specific primer and probe for HPV-16,

(5′-CCGGACAGAGCCCATTACAAT-3′) was forward primer sequence and (5′-ACGTGTGTGCTTTGTACGCAC-3′) for reverse primer and probe sequence was (5′-TGTTGCAAGTGTGACTCTACGCTTCGGT-3′). Similarly, HPV-18 was 5′-GACTCAGAGGAAGAAAACGATGAAA-3′ for forward primer and reverse primer sequence was (5′-GTGACGTTGTGGTTCGGCT-3′) and probe sequence (5′-TGGAGTTAATCATCAACATTTACCA-3′). β-globin gene 5′-GACAGGTACGGCTGTCATCA-3′) for forward primer and (5′-TAGATGGCTCTGCCCTGACT-3′) for reverse primer and probe sequence was (5′-CTAGGGTTGGCCAATCTACTCCCAG-3′).

Statistical analysis

The data were analyzed through GraphPad Prism 5.03 (Statistical package) software. Used *t*-test for *P*-value with confidence interval (CI) 95%.

Results

Overall prevalence of HR-HPV was detected to be 16.75% (33/197) and 83.25% (164 out 197) negative results reported in the studied subjects. The results analysis of HPV-16 and HPV-18 reveals in which prevalence of HPV-16 was 14.21% (28/197) and HPV-18 was detected 2.53% (5/197) in studied subjects in both groups. In the FSWs study group, overall HPV prevalence 14.85% (15 out of 101 samples) in which HPV-16 strain was detected in 12.87% (13 out of 101 sample) and HPV-18 strain detected in 1.98% (2 out of 101 sample). In the MW group, HPV-16 was prevalent in 15.62% (15 out of 96 sample) and HPV-18 in 3.12% (3 out of 96 sample) detected. There was no significance found in prevalence of HPV DNA between FSWs, that is, multiple sex partner 14.85% and MW, that is, single sex partner 18.75%. HPV 16 genotype strain (FSWs vs. MW: 12.87% vs. 15.62%) and HPV-18 genotype strain (1.98% vs. 3.12%) [Figure 1]. Study showing almost similar result in between having multiple sex partners and single sex partner. Similar result behind it may genital hygiene rather than multiple sex partners. FSWs may be high attention on sexually transmitted virus and maintaining good personal hygiene. Our study group, 7.14% (5/55) HPV infected who cleaning genital part after urination in FSWs and MW. About 22.04% (28/127) HPV infected who not cleaning genital part after urination in MW and FSW [Table 1]. In part of sexual behavior, only 6.32% (5/79) HPV16/18 infected who cleaning the genital part after sexual intercourse in FSWs and MW group [Table 1]. About 23.33% HPV-16 and HPV-18 was infected who not cleaning genital part after sexual intercourse [Table 1]. Statically analyzed used by GraphPad *t*-test for *P*-value.

Age of participants between 17 and 52 year where one very interesting to be noted in our study that the prevalence of HR-HPV was the highest in age group 17–22 year who is the youngest subject in FSWs and MW and decreasing trend was observed with increasing age, more than 40 years age was not detected HPV infection in our both study group, that is, FSWs having multiple sex partners and MW having single sex partners [Figure 2].

Table 2 showing education level, marital status, and daily income in both study group, that is, FSW and MW.

As shown in Table 2, education level revealed that the most participants (42.6%; n = 43) in FSWs and (43.8% n = 42) participants were uneducated in MW, 29.7% (n = 30) in FSWs and 37.5% (n = 36) in MW were completed high school, 24.8% (n = 25) in FSWs and 12.5% (n = 12) in MW were passed with senior secondary school level, and only 3% (n = 3) in FSWs and 6.3% (n = 6) in MW had well educated with graduate and above. About 24.8% (n = 25) of the studied group of FSWs were single/unmarried, 47.5% (n = 48) in FSWs and (85.4% n = 82) in MW were married, 18.8% (n = 19) in FSWs and 7.3% (n = 7) in MW were married but separated with or without legal separation, 1% (n = 1) in FSWs and 2.1% (n = 2) in MW were divorcee, and 7.9% (n = 8) in FSWs and 5.2% (n = 5) in MW were widowed. Most of the participants had max. daily income ≤500 (in Indian Rupees) (US$6.71) 55.4% in FSWs and 50% in

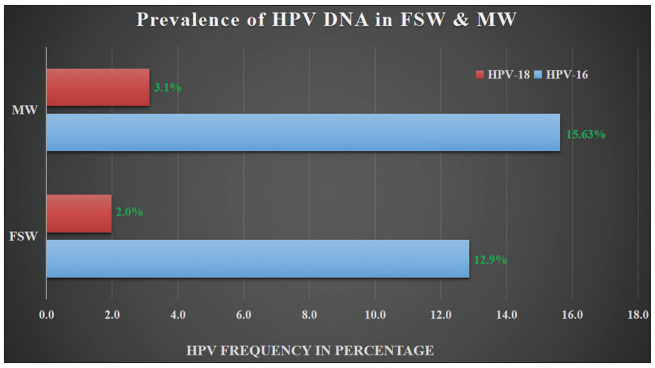

Figure 1: The graph showing frequency of HPV-16 and HPV-18 strain was almost similar HPV infection in FSW and MW. FSW: Female sex worker, MW: Married women

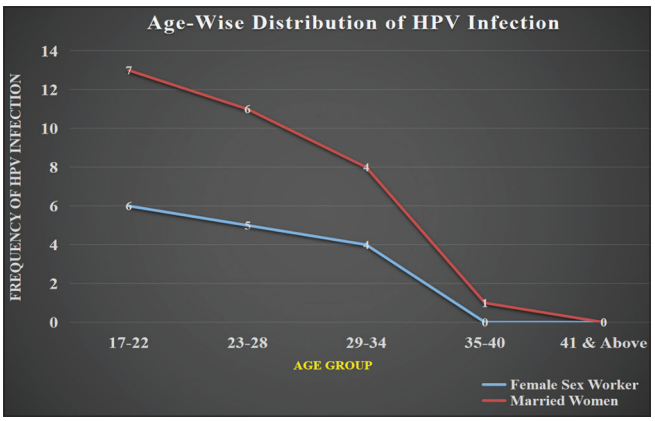

Figure 2: Age wise distribution of HPV infection in FSW and MW. As graph shown higher infection with young age group (17–22 years), (23–28 years), (29–34 years), and decreasing HPV infection with old age (≥40 years)

Table 1: Different characteristics of the women from the FSWs and MWs included in the study group

Variables	FSW (*n*=101)	MW (*n*=96)	*P*-value
	n (HPV 16/18)	*n* (HPV 16/18)	
Age-group			
17–22	35 (6)	33 (7)	0.0004
23–28	32 (5)	28 (6)	0.0003
29–34	21 (4)	25 (4)	
35–40	10 (0)	8 (1)	
≥41	3 (0)	2 (0)	
Total	101 (15)	96 (18)	<0.0001
Education level			
Uneducated	46 (8)	42 (10)	<0.0001
Up to high school	36 (6)	37 (7)	<0.0001
Up to senior secondary school	15 (1)	12 (1)	<0.0001
Graduate and above	4 (0)	5 (0)	NA
Age at first sexual intercourse (years)			
≤18	52 (8)	53 (10)	<0.0001
19–23	47 (7)	42 (8)	0.0003
≥24	2 (0)	1 (0)	
Genital hygiene			
Cleaning after urination	55 (2)	15 (3)	<0.0001
Not cleaning after urination	46 (13)	81 (15)	<0.0001
Cleaning during menstrual period	60 (4)	2 (2)	<0.0001
Not clean during menstrual period	41 (11)	72 (16)	<0.0001
Sexual Behavior			
Cleaning genital part after intercourse	72 (4)	5 (1)	<0.0001
Not cleaning genital part after intercourse	29 (11)	91 (17)	<0.0001
Partner use condom	69 (6)	42 (8)	<0.0001
Partner not use condom	31 (9)	54 (10)	<0.0001
Age at marriage (years)			
≤18	79 (13)	84 (14)	<0.0001
≥19	22 (2)	12 (4)	<0.0001
Age at first pregnancy (year)			
≤20	63 (10)	70 (14)	<0.0001
≥21	22 (2)	12 (3)	<0.0001
Number of pregnancy (*n*)			
≤2	44 (5)	31 (6)	<0.0001
≥3	57 (10)	65 (12)	<0.0001

MW, followed by Rp 5001–999 (33.7%) in FSWs and 40.6% in MW and ≥1000 (10.9%) in FSWs and 10.4% in MW. The majority of FSWs joined as a profession voluntarily, some FSWs were constrained to join in this profession indirectly or directly by their family member/spouses.

Table 3 showing HR-HPV infection duration of sexual exposure in FSW and MW.

As shown in Table 3, FSWs and MW, age ≤19 years had highest HPV infection (21.3%), followed by those age 20–29 years (17.7%) and those aged 30–39 years (9.8%), ≥40 years has no infection detected in my study group. About the duration of sexual activity, the highest HPV-16 and HPV-18 was observed in FSWs and MW with a duration of ≤2 years (35%), followed by 3–7 years (30.8%), 8–12 years (1.8%), and 13–40 years (4%).

Different characteristics of the women from the FSWs and MW included in the study group

Table 1: This table based on asked this question from FSW and MW population. Questionnaire based on age group, genital hygiene, sexual behavior, age at first intercourse, age

Table 2: Distribution of education level, marital status, and daily income in FSW and MW

Characteristics	FSW (*n*=101)	MW (*n*=96)
	n (%)	*n* (%)
Education level		
Uneducated	43 (42.6)	42 (43.8)
Up to High School	30 (29.7)	36 (37.5)
Up to senior secondary	25 (24.8)	12 (12.5)
Graduate and above	3 (3.0)	6 (6.3)
Marital Status		
Single/Unmarried	25 (24.8)	NA
Married	48 (47.5)	82 (85.4)
Separated	19 (18.8)	7 (7.3)
Divorcee	1 (1.0)	2 (2.1)
Widow	8 (7.9)	5 (5.2)
Maximum Income (Rupees/day)		
≤500	56 (55.4)	48 (50.0)
501–999	34 (33.7)	39 (40.6)
≥1000	11 (10.9)	10 (10.4)

Table 3: Age of initiation and duration of sexual activity in FSW and MW and corresponding to HR-HPV status

Age of initiation (years)	No. of sample (*n*=197)	% with HPV 16/18
≤19	75	16 (21.3)
20–29	62	11 (17.7)
30–39	51	5 (9.8)
>40	9	0 (0)
Duration of sexual activity (years)		
≤2	40	14 (35.0)
3–7	52	16 (30.8)
8–12	55	1 (1.8)
13–40	50	2 (4.0)

at marriage, age at first pregnancy, and number of pregnancies in study group. It is used GraphPad software test for *P*-value.

Discussion

Most of the sexually exposed/active women infected with HPV at least once in their life.[18] It is presumed and accepted that sexually active/exposed women will have occurred HPV infection at least once in their whole life. In this study revealed that oncogenic HPV prevalence in MW was high (18.75%). Oncogenic HPV prevalence in FSWs (14.85%) did not differ significantly from HR-HPV prevalence in MW who has single sex partner. This study investigated that FSWs more aware of STD and focused on high genital hygiene as well. They were also more attention, knowledge and aware their cervical screening than MW. In addition, we observed that HR-HPV positive FSWs were younger age than HPV negative in FSWs or MW. According to Soohoo *et al*., statistical meta-analysis published about perspective of uteri cervix HPV infection among FSWs on 35 papers found eligible for review. Overall, median of HPV prevalence was reported 2.7% (range between 2.3% and 100%).[19] Similar study conducted in Netherland and Spain used HPV assay as in our study and there was no significant difference in HR-HPV prevalence (Curacao: 25%, $n = 76$; Oviedo Spain 27.8%, $n = 187$, $P = 0.64$).[14,20] During the interview from the participants, we observed the image that the genital hygiene, sexual behavior, and awareness level of primary and secondary STD prevention and former STD were notably higher in FSWs than MW. We obtained a higher history of STD reported in MW as compared with FSWs. These outcomes confirm poor genital hygiene, lack of sexual behavior, and awareness of Uteri cervix malignancy prevention strategies under MW.

Novelty of our study that it is the first study conducted in Bihar, India, to report HR-HPV prevalence of FSWs and MW in healthy population with using non-invasive method urine sample by RT-PCR technique. Non-invasive urine sample is easy to collect from participant, cost effective and can cover mass level of screening for cervical cancer. Our study has also some limitation; small sample size of FSWs ($n = 101$) in our research.

The prevalence of HR-HPV infection was detected to be 16.75% (33/197). It is appearing that HPV-16 was most prevalent HPV strain 14.21% (28/197) in this population, which detected either single or in combination with another HPV genotypic strain. This was followed by HPV-18 only 2.54% (5/197) in both study population. Trend pattern between HR-HPV prevalence and age (17–52 years) with this group; (17–22 years) statically significant ($P = 0.0004$), (23–28 years) ($P = 0.0003$) found which statically significant value (<0.0005) [Figure 2]. Definitely, HR-HPV prevalence in FSWs and MW was peak among young age (17–22 years) [Figure 2] and decreasing with middle aged group such as (29–34 years), (35–40 years), and disappear of HPV infection in (>40 years) age group women, explaining number of regions [Figure 2].[8,21,22] Similar observation was found in other studies that revealed a decreasing pattern of HPV infection with older age.[5,23] This can be cause of acquired immunity, which perhaps clean the virus in older women (>40 years) in both study group. Decrease HR-HPV DNA in old women agreed with other studies that argue in favor of biological effect, such as increased immunity to HPV with age.[5]

These results compared with some other research as; a study conducted in West Bengal state of India in 2008, overall prevalence of HR-HPV was detected 25% in FSWs in which HPV-16 was 10% and HPV-18 was 7% prevalent in FSWs.[5] Another similar study conducted among FSWs in Tago, Sub-Saharan Africa in 2019, the frequency of HPV-16 was 10.7% and HPV-18 was found to be 7.1%.[24] A similar study conducted on FSWs and non-FSWs in Curacao in 2018, its reported overall prevalence of HPV was 25.0% detected in FSWs and 29.4%

detected in not FSWs women population whereas HPV-16 was 7.9% in FSWs and 3.9% detected in non- FSWs, HPV-18 was found to be 3.9% in FSWs, and 3.1% in non- FSWs women.[14]

The study observed association between HPV infection and education or marital status in Table 1. Statistical significance ($P < 0.0001$) is high in uneducated subject women of HPV infected. About age at initiation of sexual activity, showed that HR-HPV prevalence was 21.3% (16/75) at aged ≤19 years group, 17.7% (11/62) in 20–29 years group, and 9.8% (5/51) at age group 30–39 years. We observed that genital hygiene such as cleaning after urination, cleaning during menstrual period, and cleaning after sexual intercourse statistically significant ($P < 0.0001$) [Table 1]. Sexual behavior, uses of condom with sexual partner, age at first pregnancy, number of pregnancies was also statically significant ($P < 0.0001$). This indicate that transmission of HPV infection more infection in MW than FSWs. Sexual behavior mainly early age of first sexual debut and numbers of sexual partners during life time has been identified as a significant risk factor for acquiring HPV infection and progression of cervix carcinoma in addition to history of infection with other high parity, STD, and cigarette smoking.[25] The probability of genital hygiene rather than multiple sexual partners stands more apt as a cause of HPV infection. Because of maintained good personal hygiene during and after sexual activity, washing after urination and cleaning properly during menstrual period. It can be explained by the higher standard of self-care such as genital hygiene, cleaning after urination, cleaning duration menstrual period, cleaning after intercourse, and use of condom. "Male factor" can be explained the HR-HPV exposure in women.[11,16] Our subjects were asked about the number of recent daily sexual partner as this is considered to be a better predictor of the prevent and incident HPV infection than the number of lifetime sex partners.[11,16,17] We observed that significant association between the more promiscuous sexual behavior of FSWs and higher prevalence of both high risk and multiple HPV. Moreover, high number of sex partner high number of vaginal sex partner, the high frequencies sexual intercourse, and practicing anal sex have been significantly related to an increased risk for HPV infection[17] as well as shorter time interval between a new partner and engaging in sexual intercourse.[11,17] About study associated with poor genital hygiene as significant co-factor in progression of cervix cancer as a consequence of previous HPV infection[17,26] in developing countries. A study conducted in India, poor hygiene associated with increased risk of HPV infection, but not with ICC risk among HPV positive women.[17,27] Some study conducted in developing countries, high risk of STD among women, observed that women who generally douche after sexual intercourse, genital warts are actually less common.[17] Other observation found that no direct relation between vaginal cleaning practice and risk for STD such as HPV infection,[28] though some recent paper associate regular vaginal douche after sexual intercourse as risk factor for HPV infection[29] or for cervix carcinoma.[30] Hence, the assessment of vaginal douching and other genital part cleaning practice in inconsistent in the prevention of HPV infection.

A correlation has been observed between HR-HPV prevalence in penis and HPV related cervical neoplasia in female partner.[22,31] It has been pointed out that in the absence of screening program, a women's risk of cervix carcinoma can depend less on her own sexual behavior than that of her male partner.[22,32] There is an argument that condom may not entirely covered the infected areas thus rendering insufficient protection against HPV infection.[17] In our study, MW showed significantly a higher rate (18.75%) of HPV infection. The reason for higher prevalence of HPV infection in MW may be due to their different lifestyle, sexual behavior, and lack of awareness. Genital hygiene shown presence of primary menarche (≤12 years) with high sexual permissive and multiple number of sex partner contributes to multiple pregnancies, higher parity, malnutrition, and immune deficiencies[33] leading to increased HPV infection. It is observed that increased risk of HPV infection in MW only after exposure of sexual activities. It is possible because of immature genital organs of MW are more susceptible for contracting infections.[34] Among many correlations of low socio-economic status, we found association with uteri cervix risk in Chennai (e.g., il literateness, poor nutrition, under-weight, and paan-chewing) [35] indicator of poor hygiene condition exhibited strong effect. Poor hygiene condition plays an important role on risk of HPV infection and uterine cervix carcinoma has been reported.[26] Although, higher prevalence of HPV infection was associated with poor hygiene condition among control women, it is not increased with ICC risk among HPV positive women.[27] Paan chewing was 18.5% of cases reported and 7.6% reported in control women where smoking was reported very rare.[35] Chewing was most common in less illiterate women but two-fold increased ICC risk was found after adjustment of occupation, education, and some other major risk factors. Various studies have revealed a relationship between sexual activity indicators and ICC risk[36] as expected since the essential cause of the disease, HPV is sexually transmitted. Although, women reported two or more lifetime sexual partner at very small proportion and early age of first sexual debut or early age of marriage were not significantly related to carcinoma risk after allowance for socio-demographic and reproductive factors. When we did, role of education, marital status, high parity, early menopause and a woman reported of her husband's extramarital sexual relationship were similar to those obtained without such restriction. Conversely, the apparent effect of poor hygiene condition disappeared.

It is clear from the questionnaire that almost 80% of girls who have had their partner or boyfriends showed higher prevalence of HPV infection. There may be several other causes for HPV transmission from mother to offspring,[37,38] non-penetrative sexual practices and HPV transmission via fomites. HPV has also been detected in fingernail and fingertip sample of young sexually exposed women,[39] which might have transmitted during washing of genital area.

Hygiene and cleanliness were very poor during menstrual period; as such they repeatedly use old cloth along with cow dung ash and other non-hygienic material in their sanitary pad. One general approach focused on urine sampling to promote to be highly sensitive and specific for true uterine cervix malignancy, thus reducing costly effect over treatment and mass level of screening program. In this study, we also investigated worth of urine specimen to detection of HR-HPV DNA strain with FSWs and MW in Bihar, India.

Urine specimen used as a non-invasive method to detection oncogenic HPV DNA for cervical cancer. Urine sample happy to collect from women, less expensive form of sample collection, easily agree to participate and maximum reach out to the population for screening program that bypasses the medical examination and even easier to perform than cervical swab.[40,41] While Cervical swab/Vaginal swab could not be ease to sampling from women, not simply agree to participate in the screening program, need of gynecologist, and certified nurses and high expenses. Cervical specimen panics to sampling because sterile specula injected in the vagina, rotating its 2–3 times on cervix in the uterus. Approach of urine specimen should facilitate HPV detection in females who do not have access to gynecologist and should be more attractive to women whatever their social origin. Consequently, the screening program of cervical cancer optimum coverage could be increased considerably, primarily by reaching population in limited resource setting. We show HPV DNA assay in urine specimen could absolutely replace cervical swab/vaginal swab. Therefore, urine specimen has chosen for this study. In recent times, meta-analysis reported that detection of HPV DNA assay in urine specimen has truthfulness accuracy for the presence of cervix HPV.[42] Urine specimen was found to have high accuracy when compared with detection in vaginal swabs, with sensitivity 77% and specificity 88%.[42] However, small number of studied evaluated on the basis of clinical performance of urine-based HPV DNA assay in the prediction of cervix precancerous lesions and cancer dysplasia.[42,43] About the use of clinically validated HR-HPV DNA assay in urine samples has limited information.[44,45] The great sensitivity and specificity of HPV DNA assay in urine specimens with comparison to cervical swab HPV testing found in meta-analytical study and it suggested that HR-HPV DNA in urine has potential to use in case of cervical dysplasia screening.[42]

This study described herein a greatly sensitivity linear made millions copies of HPV DNA by RT-PCR which used to diagnosis of HPV infection. The number of HPV genotypes strain was detected higher in urine specimen by RT-PCR. High rate of genotyping recommended that HPV genotyping in urine reflect actual infection in cervical tissue. The percentage agreement between cervical swab and urine sample was 88% using HPV DNA detection kits.[44] All of genital HPV infection cannot be detected using urine sample but HPV DNA assay provide substitute for use in epidemiological screening in which invasive specimen is difficult to perform. Under these situations, using urine for HPV DNA offers a distinct advantage.[46] The previous study has compared HPV detection rates between cervical and urine samples to evaluate the ability of urine-based assay to detect the prevalence of HPV independently of cervical cytology assays.[47,48] Evidence advice that the sensitivity of urine test for HPV-16 and HPV-18 was higher for participants with uterine cervix carcinoma (88.8%) than for those with high and low-grade lesions.[47] Alternatively, certain urine specimen may be yield low efficiency DNA amplification due to occurrence of inhibitory ingredients in the urine or HPV DNA loss during processing.[49] Several model-based cost-effectiveness analyses suggest that HPV DNA screening program in certain high risk dense population is the most effective methodology to control cervical cancer.[50]

Conclusion

The study indicates that oncogenic HPV prevalence did not different significantly in FSWs and married (single sex partner) women. FSWs having higher awareness about genital/personal hygiene for sexually transmitted virus than MW. FSWs maintained good genital hygiene rather than MW. FSWs may be washing practice of genital organ with water after every sexual activity. The probability of genital hygiene rather than multiple sexual partners stands more apt as a cause of HPV infection. Hence, genital hygiene reduced the burden of HPV infection in women. More study is required for the significant validation behind the cause of transmission of HPV. This study will give new direction to the etiology and high incidences of cervical cancer. More study is required for the significant validation behind the cause of transmission of HPV. Strength of the study is significant for health policy maker to implement prevention strategies of cervical cancer. This study advises to develop more awareness program about genital hygiene in women to reduce the HPV infection. We also advise to Bihar government to develop mass level screening program through non-invasive method urine sample used for RT- PCR for detection of HPV infection in women is reliable, specific, and sensitive and highly acceptable for the participant.

Authors' Declaration Statements

Ethical clearance

Ethical clearance was obtained from "Ethics Committee Mahavir Cancer Sansthan and Research Centre" Patna India for urine specimen of the Subjects. Reference Number is MCS/Admin/2018-19/1223 as on dated 23/08/2018.

Consent to participant

Consent was taken from participants and the objective and details of the study was explained.

Consent for publication

All the authors of the study provide their consent for publication.

Availability data and materials

The data of this study are available and will be provided by the corresponding author on a reasonable request.

Competing interest

None to declare.

Funding statement

There were no funds received for this study.

Authors Contribution

Mohammad Ali and Akhtar Parwez designed this study, Akhtar Parwez, Rahul Kumar and Sristy Kumari collect the sample and perform lab work. Sunit Singh verified and analyzed the methodology, Mohammad Ali and Arun Kumar supervised the finding of this work. Akhtar Parwez took the lead in writing manuscript. All authors discussed results and contribute to final manuscript.

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