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Cervical screening and assessment of risk factors for Human papilloma virus infections among women attending a tertiary care hospital

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***Abstract*---**Aim: To assess the presence of HPV among women who were visiting gynaecological out patient department for any cervix related reason. Material and Methods: Cervical scrapings were collected using Cytobrush. Pap smear results were collected from the pathology department and Histopathological confirmation for high grade lesions was also collected. PCR has been performed to look for the presence/absence of HPV DNA in all the samples by using MY09/11 primers. Results: Risk factors responsible for acquiring HPV infection were examined. Out of 60 Cytobrushes samples 32 samples had squamous intra epithelial lesions, out of which 16 were again Histopathologically confirmed as malignant cases. Chronic cervicitis were seen in 16 samples. Whereas, 8 were inflammatory smears and 20 were Negative for Intraepithelial Lesions. Only 16 HPV positives were confirmed by PCR. Conclusion: This study showed the incidence of HPV is very low in contrast to other studies in India.No awareness of HPV infections has been found in our region either. However, these findings could have important connection in the prevention of cervical cancer.

***Keywords*---**HPV, HPV DNA, Kolar, Socio - Demographic, Cervical Carcinoma.

Introduction

Cervical carcinoma is one of the major health issues for women living in the region of India & every year almost 120,000 women suffer from this disease. Available data states India records 15.2% deaths of cervical carcinoma among the

world cervical cancer deaths. It is the 3rd most widespread cancer in women worldwide ranking first among developing countries with approximately incidence of 5,69,847 & 3,11,365 deaths in 2018 as reported.[1]

Overall, Most developing countries reports four times higher cases of cervical carcinoma than industrialised countries. Mupetti et al. in their study states developing countries reports 80-85% of cervical cancer deaths.[2,3] HPV was recognised as the main causative agent of cervical cancer but disease can only develop/ progress if there is persistent HPV infection of the cervical epithelium.[4] The literature has shown that HPV oncogenic types (16,18,31,33,35,39,45,51,52,56,58,59,66) can occur up to 99.7% of cervical cancers.[5]

HPV has a size of ~8kb with double stranded DNA genome encoding for L1 and L2 2 late genes and (E1, E2, E4, E5, E6, E7) 6 early genes. Integration occurs at E2 region with a complete or partial disruption of its open reading frame. E6 and E7 are the two oncogenic genes which are repressed by E2 but in the absence of E2 this transcriptional control is lost which results in overexpression of E6 and E7. This leads to immortalisation or apoptosis and transformation of cells. E6 and E7 are the major oncogenes that play a significant role in progression of cancer. E6 aletrs DNA repair, inhibits apoptosis and destabilizes the chromosome when it binds to the tumour suppressor gene P53. E7 binds against the retinoblastoma protein (Tumour suppressor) and enhances dysregulation of the cell cycle. In addition, oncoprotein E7 also interacts with several other cellular proteins, is involved in inhibiting apoptosis and by-passing immune surveillance functions.[6,7,8] According to literature sexual activity plays the most important role in acquiring HPV infection.[9,10]

The factors such as age, marital status, educational level, and employment status and income level influence the screening practices of women and have been studied by many researchers interested in advancing cancer screening.[11]
Regular cytological examination of the cervix in all sexually active women can prevent the occurrence of cervical cancer. A review of guidelines and recommendations for the early detection of cervical cancer suggests that screening should commence few years after sexual activity begins. A woman does not need to be screened after a total hysterectomy except the cause for the surgery was cervical carcinoma. [12]

In addition, awareness of genital hygiene and visiting hospitals in pre-clinical phase help control cervical cancer in urban settings. The prevalence of cancer is alarming in the rural population where the majority of women are illiterate and unaware of the factors that contribute to the development if cervical cancer.
Detection of precancerous stage by early screening can prevent cervical cancer with simple treatment. The most internationally accepted and economical screening test is pap, reported by Hawkins et al.[13] Despite the availability of pap tests the incidence of invasive cervical carcinoma remains high, especially in rural India .[14]

Although HPV vaccines have recently been introduced, they only prevent infection with the main HR HPV types. However, cervical cancer can also be caused by

other genotypes of this virus as well. We must therefore continue to rely on early detection of infections by screening methods and furthermore there is no literature of the circulating genotypes of HPV in women in our locality. Therefore, the present study aimed to screen women attending OBG OPD in our tertiary care hospital with gynaecological problems or abnormalities for the presence of HPV DNA in order to understand the circulating genotypes of HPV in these patients.

Materials and Methods

For this prospective study total 60 subjects were enrolled after obtaining ethical clearance from institutional ethics committee. This study was conducted between August 2019 & April 2020. Data and sample collection were done at the Department of OBG and sample processing was done at the Department of Microbiology, Sri Devaraj URS Medical College, Kolar.

Inclusion criteria:

- Married women,
- Non pregnant and
- Those who had not undergone hysterectomy were included.

Exclusion criteria:

- Pregnant Women,
- Those who refused to sign the consent were excluded.

Data collection

Cytobrush samples were collected from 60 subjects who visited OBG OPD with any gynaecological complaints. Cytobrush samples were taken from the patients before taking a pap smear as a routine procedure. Cytobrush samples were collected which were after collection placed into pre chilled phosphate buffered saline and stored at - 20°C until DNA extraction, not more than four weeks. For patients with cytology report stating High grade squamous intraepithelial lesions a small punch biopsy was taken and sent for histopathology confirmation.

Extraction of DNA

DNA was isolated from cytobrushes using Qiagen DNA Mini kit (Qiagen GmbH, Hilden - Germany) following kit literature and extracted DNA was stored at -80°C until PCR for HPV DNA detection.

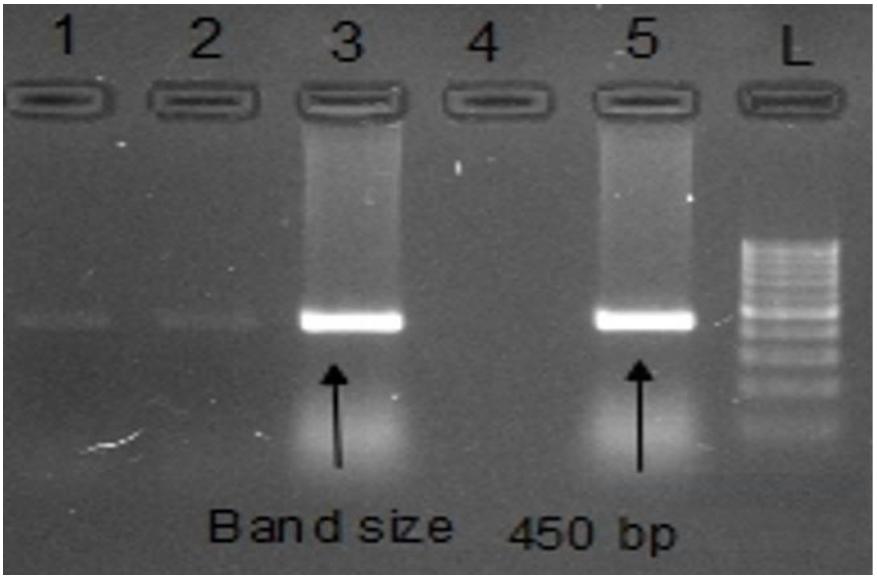
HPV detection by Polymerase Chain Reaction (PCR)

DNA amplification was done by using primer sets MY09 [F5'-CGTCCMARRGGAWACTGATC-3] and MY11 [R5'-GCMCAGGGWCATAAYAATGG-3'] targeting L1 gene with a band size of 450 base pair.The oligonucleotide primers were procured from the Sigma Aldrich Private India Limited, Bangalore. PCR was performed to detect HPV DNA. A 25 µL reaction was assembled that contained 3 µL sample DNA, 10 µL Ampliqon Red 2X Master mix (Tris-HCL pH 8.5, Ammonium sulphate (NH4)2SO4, 0.2 unit/ µL Ampliqon Taq DNA polymerase, 0.4 Mm deoxynucleotide triphosphate (dNTPs), 3 mM MgCl2, 0.2% Tween 20), 1

µL of 10 pmol of each primer (forward and reverse) and 10µL molecular grade water. The PCR condition for each primer set is given in [Table-1]. The amplicons were then analysed on 2% agarose gel, which is stained with Ethidium bromide and visualized on a UV trans-illuminator and photos of bands were recorded using gel documentation system (Major science, United states) as shown in [Figure-1]

[Table - 1]
Polymerase chain reaction (PCR) conditions for primer set

Primer	Initial Denaturation	Final Denaturation	Annealing	Extension	Final Extension
		35 cycles			
MY09/ MY11	95 ° C for 5 min	95 ° C, 30sec	55 ° C, 1 min	72 ° C, 30 s	72 ° C, 5 min



[Figure-1]

PCR amplicon on agarose gel showing HPV DNA. L is 100 base pair (bp) DNA ladder, 3 and 5 - Strong positive (band size 450bp), 1 and 2 - Weekly positive and 4 Negative for HPV

Statistical Analysis

Windows program; SPSS 22.0 was used to perform statistical analysis. Chi square test was used for socio-demographic factors association and variables were compared between positive and negative predictive values of histopathological/cytological status of the patients. P-value < 0.05 was considered significant.

Results

Among 60 Cytobrush samples, 32 samples had squamous intra epithelial lesions,out of which 16 were again Histopathologically confirmed as malignant cases. Among which 1 had Cervical intraepithelial neoplasia (CIN) II, 5 Non Keratinizing Squamous Cell Carcinoma, 4 Keratinizing Squamous cell carcinoma, 1 Adenocarcinoma and 5 Moderately Differentiating Squamous cell carcinoma. chronic cervicitis were seen in 16 samples. Whereas, 8 were inflammatory smears and 20 were Negative for Intraepithelial Lesions/Malignancy

PCR results confirmed 16/60(26.6%) HPV positives. The occurrence of HPV in the present study among invasive carcinoma cases was high i.e., 8/16(50%) as compared to inflammatory smears 3/8(37.5%), Chronic cervicitis 2/16 (12.5%) and NILM 3/20(15%). HPV -DNA prevalence in women with different cervical abnormalities is given in [Table-2]

The HPV prevalence in the current study is low i.e., only 26.6%.
Evaluation of positive and negative predictive values for HPV DNA from 16/60 Histopathologically positive and 16/60 Histopathologically negative was done. Similarly, Evaluation of the positive and negative predictive values for HPV DNA from 8/60 cytological (PAP smear) positive and 20/60 cytologically negative cytobrush samples were evaluated as given in [Table-3].

[Table -2]
HPV-DNA prevalence in women with different cervical abnormalities

Histology/Cytology of samples	Number of samples	HPV DNA Positive
Invasive cervical cancer		
NKSCC	5	3
KSCC	4	2
ADC	1	0
MDSCC	5	2
CIN II	1	1
Chronic Cervicitis	16	2
Inflammatory smear	8	3
NILM	20	3

NKSCC-Non-Keratinizing Squamous Cell Carcinoma, KSCC-Keratinizing Squamous Cell Carcinoma, MDSCC-Moderately Differentiating Squamous Cell Carcinoma, CIN-II-Cervical Intraepithelial Neoplasia-II, NILM-Negative for Intraepithelial Lesion/Malignancy, Significance

[Table-3]
Comparison of histopathological/cytological findings of tissue biopsies and cytobrush samples with PCR findings

Total samples	PCR positive samples	PCR negative	+ ve Predictive value	-ve predictive value	p-value
Histological +ve samples (N=16)	8	8	50	80	0.0298
Histological -ve samples (N=16)	2	14			
Cytological positive samples (n=8)	3	5			
Cytological negative samples(n=20)	3	17	37.5	85	0.246

Assessment of Risk-factors associated with HPV:

Patient information sheet was provided to all the participants at the beginning of the study which was approved by the institutional ethics committee. Informed consent has been taken from all the participants. Socio demographic details of the participants are shown in [Table-4]

[Table- 4]
Socio demographic details of the participants

Age group (Years)	No. Of patients	percentage
20-30	8	13.3
30-40	16	25
40-50	17	28.3
50-60	15	25
> 60	4	8.3
Parity		
<3	14	23.3
>3	46	76.6
Age at first intercourse		
<20	44	73.3
>20	16	26.6
Socioeconomic status		
Middle	17	28.3
Low	43	71.6
Menopause		
Pre	41	68.3
Post	19	31.6

Discussion

Cervical cancer incidence in India is quite high. So early screening of HPV can be a better solution to this question. [15] HPV 16 & HPV 18 are the common genotypes. There are other High risk HPV genotypes also. Persistent infection with these HR HPV genotypes increases the risk of cervical intra epithelial Neoplasia or invasive cervical Carcinoma .[16] Various studies in the region of Kolkata, south India and Delhi reported the prevalence of genotype HPV 16 an HPV 18 high among all the genotypes causing the cervical carcinoma. [17,18,19,20]

In the present study 17/60(28.3) belongs to 40-50 years age group followed by 16/60(25%) in 30-40 years age group which correlates with a study from China had maximum number of participants in the age group of 31-40 years .[21] The occurrence of HPV in the present study among invasive carcinomas was found to be high i.e., 8/16(50%) as compared to women with chronic cervicitis and NILM being 12.5 and 15% respectively. In present study, the results of histopathological positive/negative and PCR positive/negative tissue biopsy samples were significantly correlated (P-value 0.0298) whereas; results of cytological positive/negative and PCR positive/negative for cytobrush samples were not significantly correlated (p-value 0.246).

However, the total HPV prevalence in present study was low (26.6%), In a study done by Rashmirani Senapati et al., in the region of odisha region reported the HPV prevalence was 60.33%. They also reported HPV prevalence was 93.80% in invasive cancer, 54.32% in inflammatory smears and 19.11% in cases with cervix showing normal cytology .[22] Study results of Varsha Saxena et al., also reported the HPV prevalence as 18.8% only.[23] Results of Varsha Saxena et al., and Rashmirani Senapati et al., correlated with low HPV prevalence in their study. A study done by Chakravarty J et al., from East India reported the overall prevalence of HPV among HIV positive subjects was 26.85% which according to the author was the high prevalence as compared to the general population .[24]

Results of the present study showed that 43/60 (71.6%) of the study participants belonged to the below poverty line status. One of the major risk factor found in our study was high Parity>3 which is in correlation with a study done by Munoz et al. The high number of pregnancies is may be due to early marriages in India. It has been reported that high parity leads to four fold progression of squamous cell carcinoma. In parallel to other studies, low socioeconomic status was also found to be one of the significant factor associated with cervical cancer in our study population. Rashmirani Senapati et al., study results also shows risk factors such as Age above 45 years, parity more than or equal to 3, low socio-economic situation , rural residence and postmenopausal status have significant association with HPV infection

PAP smear is a simple, cost-efficient and sensitive tool for detecting of premalignant and malignant changes in the cervix. The efficiency, sensitivity and specificity of the Pap smear depends on frequency of cervical cancer screening programs for women, adequate sample collection, and the quality of laboratory analysis .[25]

Conclusion

In this study the prevalence of HPV is low and there is completely no awareness of HPV infection. Our data suggest the need of HPV screening programmes in women belonging to age group 30-50 years, low socioeconomic group and for those people residing in rural areas. The screening programmes will help in detecting abnormal cancerous cells at their initial stage which will help in providing better treatment. Although vaccination is available for HPV, we still need to rely on early detection methods as these vaccines prevent infection by only major types of HPV whereas carcinoma cervix can be caused by other genotypes of the virus as well. Therefore, HPV molecular testing in combination with cytology should be used to identify premalignant lesions.

Limitations

Since the findings of this study relied on small number of cases, future studies with large number of cases may support significant results.

Conflicts of interest

The authors declared no conflicts of interests.

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