



Human Papilloma Virus Types 16/18 Distribution in Invasive Cervical Cancer: An Evidence for Vaccination in Bihar, India

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Authors' contributions

This work was carried out in collaboration among all authors. Authors MA, VR, KM, VT and RK designed the study. Author RK and BP performed the statistical analysis. Authors RK and RC wrote the protocol. Author RK wrote the first draft of the manuscript. Authors RK and AP managed the analyses of the study. Authors RK and AP managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

There is high incidence of cervical cancer in Bihar, India. Vaccination for cervical cancer in developed countries has played a crucial role in limiting the incidence rate of cervical cancer worldwide. In consideration of debate on clinical efficacy of Human Papilloma Virus (HPV) vaccine in India, study on the prevalence of high risk HPV 16/18 strains in different regions of the nation becomes very crucial. Few individual states have started vaccination but centralised vaccination program does not exist due to lack of sufficient genotypic study of Human Papilloma Virus in

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different parts of India. Bihar is the third most populous state of India and HPV 16/18 distribution has not been reported yet. The nationwide data of HPV 16/18 will help to develop a unified centralised vaccination program. We carried out a distribution study of high risk HPV type 16 and 18 in cervical cancer patients attending a tertiary care hospital of Bihar, India. HPV 16/18 types were analysed in cervical cancer tissues (n = 96) of patients attending the regional cancer hospital of Bihar. Tissue samples were analysed for HPV 16 and HPV 18 using a Real Time PCR technique. The results suggest very high prevalence of HPV 16/18. HPV was identified in all the samples (96/96). About, 74 (77.08%) samples presented with HPV 16 whereas, 16 (16.67%) of the samples presented with HPV 18. 6 Co-infection was presented in 6 (6.25%) of the samples of cervical cancer tissues. HPV 16/18 prevalence is more in the women aged between 41 to 61 years. We report 100% prevalence of HPV 16/18 in the cervical cancer tissue samples. A way to minimise this gynaecological concern would be to introduce prophylactic vaccines and early screening in the state of Bihar. The data generated would be crucial in drafting for community screening of HPV. We strongly emphasize the prophylactic HPV Vaccination against HPV 16 to control the alarming rate of cervical cancer in one of the most populous state of India, Bihar.

Keywords: Cervical cancer; HPV vaccine; Human Papilloma Virus; Prevalence; Bihar; HPV 16/18.

1. INTRODUCTION

As per GLOBOCAN 2018, Cancer of cervix is the 3rd most widespread universal gynaecological malignancy with an estimate of 5,69,847 new cases and 3,11,365 deaths every year. In India, about 96,222 new cases of cervical cancer are identified annually. In India, it is the second most widespread and deadly cancer following Breast cancer. About 60,078 deaths per year in India are attributed to cervical cancer. Female population at the age equal or more than 15 years is considered at risk for cervical cancer. In India, this population at risk is about 469.1 million. India has an estimate population of 1350.1 million whereas Bihar being one of the populous states is 120.4 million. The high mortality rate of cervical cancer reported is mainly attributed to low accessibility of screening programs in India. As per household survey carried out by World Health Organisation, only 5 % of women has been screened once in their life that too has been only reported in Karnataka, India [1]. It has been stated that incidence of cervical cancer is more in rural areas than compared to urban areas [2]. In Bihar, 88.7% population is living in rural area [3]. Majority of Indian population resides in rural background so, it is possible that actual mortality rate may be more than stated as many residents of Bihar are not medically insured and cannot afford medical care. To add to this notion, GDP of Bihar is 610 US \$ and 78% of the population wage was less than 1.67\$ per day in 2019 and hence the probability of under reporting is too high [4].

Human Papilloma Virus is now a well-established cause of cervical cancer [5,6,7]. It is sexually transmitted and oncogenic subsets are known as

high-risk HPV types. About 120 different HPV types has been reported which are further classified as low-risk and high-risk (HR) on the basis of association with cervical cancer [8]. Till date identified HR HPV types include 16, 18, 31, 35, 39, 45, 51, 52, 58, 59, 69 and 73 [9]. These are known to be HR HPV due to their association in almost all types of cervical cancer. HPV 16 and 18 are most frequently associated with cervical cancer throughout the world [10]. More than 70% of the cases are infected by HPV 16 and HPV 18 [11,12]. Presently existing vaccines target these two HR types namely HPV 16 and HPV 18. International Agency for Research on Cancer (IARC) working group report 6 other HPV types mainly HPV 31, 33, 35, 45, 52, and 58 mentioning that they should be prioritised when developing or assessing the current vaccines or developing future polyvalent HPV vaccines [13,14]. But this study accumulates data mainly from developed nations. In India, even the existing vaccines are not in practice.

Most of the studies reported are from developed countries and represents very few specimens from India. The incidence of diseases is alarmingly very high and the basic vaccination against HPV 16 and HPV 18 is also not in central vaccination policy. Still prevalence study of the most common HPV 16 and 18 has not been done in few of the states. Bihar is one of them where incidence of the disease is alarmingly high but no initiation in this step has been taken.

Various small-scale local studies from India presenting HPV 16/18 type distribution in association with cervical cancer have been carried out [15-32]. Not a single study has been

carried out in Bihar for HPV prevalence in cervical cancer patients where the 88.7% population resides in rural India. There remains a need to better characterize the HPV 16/18 relationship to cervical cancer particularly by the presence of more cervical cancer patients from Eastern India.

In this study, our goal was to address the lack of data by giving a pilot study to have an idea whether the existing vaccine could be utilised in the third largest populous state of India whose population is more than many countries as well. The study also compared HPV 16/18 association with Squamous Cell Carcinomas (SCC) and Adenocarcinomas (ADC) and examine the relationship of patient age to HPV detection in cervical cancer. We evaluated the prevalence of HPV subtypes 16 and 18 among women with histologically confirmed diagnosis of cervical cancer at a tertiary care hospital in Bihar, India by RTPCR.

2. MATERIALS AND METHODS

Current study was planned and conducted at Mahavir Cancer Sansthan & Research Institute (MCSRI), India. MCSRI is a tertiary care hospital which provides inpatient and outpatient services to cancer patients. Patients visiting MCSRI represent a diversified group from different states as well as different cultural and ethnic background. This study was conducted during the period of August 2017 to July 2019. A total of 96 tissue biopsy samples were collected from histopathologically confirmed cases of cervical cancer. Biopsy samples were preserved as the Formalin fixed Paraffin Embedded (FFPE block) in the pathology department of MCSRI. Biopsy samples were histologically confirmed as squamous cell carcinoma or adenocarcinoma. Most of the patients were from low socioeconomic status representing a poor immunity and hygienic status as per personal counselling. Multiparity and illiteracy was also an impeding finding as per personal interaction with the patient.

2.1 Extraction of DNA from FFPE Blocks

Sections of 5-10 μ m thick of paraffin-embedded blocks were cut with microtome. The cut sections were kept for the deparaffinization. About 1 ml of 97% xylol was added to each 2ml eppendorf tube and the mixture was then homogenized through a vortex mixer. It was then heated in an oven at 60°C for 10 minutes followed by

centrifugation at 2,500 rpm for 10 minutes. The excess ethanol and xylene was removed. The mixture was then vortexed for 15 seconds and was incubated at normal room temperature for 3 minutes. It was then centrifuged at 12,000g for 15 minutes at 4°C. Next, the pellet underwent washing twice in 300 μ l of 100% ethanol, and then 1 ml of 75% ethanol was added. The material was placed in the refrigerator for the further amplification process.

2.2 HPV DNA Detection by PathoDetect: HPV PCR Detection Kit (Type 16/18)

The deparaffinised sample was treated with about 180 micro litre of Lysis Buffer I and Lysis Enhancer buffer. After vortex and spinning, sample was incubated at 55 C. Then RNA out was added followed by binding of DNA in the Spin Column in the collection tube. It was centrifuged and followed with washing of DNA. Wash Buffer I and Wash Buffer II were respectively added with 13000g centrifugation. Finally it was transferred in Spin Column for Elution of DNA. Elution buffer was added to the column and centrifuged. The tube contained the purified genomic DNA. It was preserved at -20 C for long term storage. The sample was processed as per the Pathodetect, HPV PCR Detection kit (Type 16/18) (MylabLifesolutions) which is a Real Time PCR kit. Real Time PCR for detection of HPV 16/18 DNA was done through Pathodetect (qPCR) Protocol designed for an in vitro detection of HPV DNA from extracted DNA samples. The Mylab Solutions kit uses one step real time PCR with Taqmanfluorogenic probe chemistry which uses the 5' nuclease activity of Taq DNA polymerase and enables the detection of a specific PCR product as it accumulates during PCR cycles [29].

2.3 Statistical Analysis

The data was analysed through (Statistical Package for Social Sciences) SPSS version 16.0.

3. RESULTS

A total of 96 samples were analysed. The age ranged between 23 to 96 years with a median age of 54 years. Most of the women attending MCSRI for cancer treatment and care are from low socioeconomic strata of society with multiparity and high illiteracy based on data from hospital records.

As per Table 1, Most of the biopsy samples (89/96, 92.7%) of the tissues were of Squamous Cell Carcinoma and only (7/96, 7.2%) were of Adenocarcinoma type. Most of the patients presented in FIGO (International Federation of Gynaecology and Obstetrics) stage III. Among the biopsy samples, HPV 16 was the most prevalent strain (74/96, 77.08%), followed by HPV 18 (16/96, 16.67%). Out of 96 samples, 6 of them were co-infected (6.25%).

4. DISCUSSION

The HPV 16/18 study was carried out for the first time in the state of Bihar, India. Patients included in study were from different districts of Bihar. This study needs an emphasis as the state population is more than many small countries in the world. India is a diverse country with varying ethnicity, social diversity and cultural diversity which might result in difference in HPV prevalence in different states. So, it is required that HPV distribution of atleast 16 and 18 types for which vaccination is present must be studied in every state of the country. This study also becomes crucial due to its majority population residing in rural background and also the area is Arsenic Hit [15,16]. These two parameters make the study being carried out more crucial. It has been reported that Arsenic compromises the immunity which is associated with HPV infection and its persistence [17,18].

The available literature shows that the distribution of HPV 16/18 in women in various parts of India ranges from 9 to 94%. Incidence of HPV 16 infection along with HPV 18 in cervical carcinoma is very high as compared to other HPV type infections in India [Table 3] [33-49]. The prevalence of HPV 16/18 types found in this study is the highest. This cent per cent prevalence may be due to immune compromised status and Arsenic hit area which further makes the patient prone to infection. Results are almost similar to a study reported from Odisha, India [19]. People living in rural area are 20 times more prone to acquire HPV infection as these areas

reflect poor socioeconomic condition due to lack of access to hospitals, poor genital hygiene, poor health and lack of awareness [20].

HPV persistence is associated with risks or exposures like multi-parity, marriage with an older man and multiple sex partners [21,22]. In India, basically in northern India (Bihar), the marriage age of men are generally higher due to social and educational factors than compared to girls. Therefore, HPV vaccine as prophylaxis in the specific-aged women can curtail the gynecological malignancy burden. The biggest hurdles are the high cost of vaccines and the awareness regarding the disease and its cause in rural areas basically. A program to educate common population and health care professionals would be of great help in curbing this menace [23]. Based on the results of our study, it can be stated that awareness program, HPV testing and timely gynecological screening will be of great help in reducing the HPV malignancy burden. Spread of knowledge pertaining to the HPV, its risk factors, and its prevention to all females is pivotal in reducing the disease burden in the future [24].

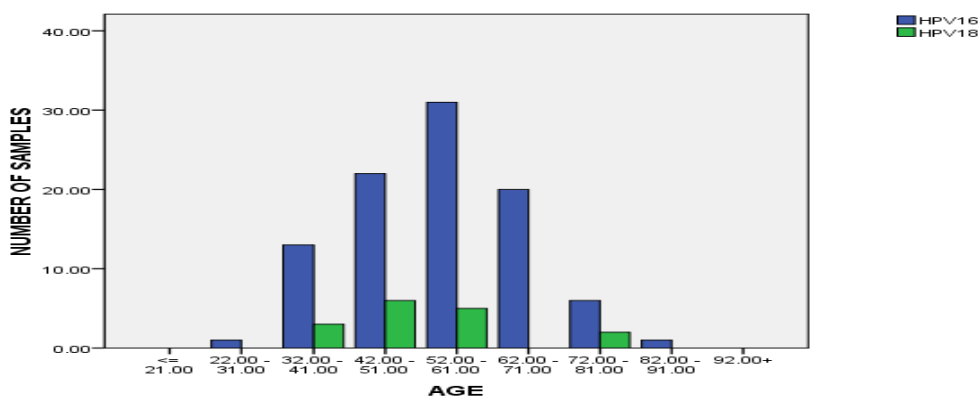
Advanced age is a crucial risk factor linked with the cervical cancer. In our study, the majority of the subjects (>50%) were in the age group of 41-61 years (Fig.1). A study performed in 2010 reported a low prevalence of HPV in the young women and general population which is in line with the findings of our study [25,26]. Though, HPV types 16/18 did not have a significant pattern in age-specific distribution (Fig. 1). Prevalence of HPV 8 has been reported lower than that of HPV 16 in India as well as others part of the world [15-30]. The prevalence of HPV 16 and HPV 18 in our study was found to be 77.08% and 16.7% respectively. HR-HPV 16/18 co-infection was identified in about 6.25% of all the cases which is in proximity to other studies conducted in India. Most of the samples (92.7%), as represented in Table 1, are of squamous cell carcinoma having clinical FIGO staging IIB and III.

Table 1. Patients histological and clinical presentation

Patient histological presentation		Frequency (%)
Histological Type :	Squamous Cell Carcinoma	89 (92.70)
	Adenocarcinoma	07 (07.20)
Stage	II B	49(51.04)
	III	45 (46.88)
	IV	02 (02.08)

Table 2.The estimated prevalence of HPV16/18 in cervical cancer

Number	Percentage	Total
Sample Size	96	
HPV Negative [16/18]	0	0
HPV Positive [16/18]	96	100
HPV types		
Single infection		
HPV 16	74	77.08
HPV 18	16	16.67
Multiple infections		
HPV 16/18	06	6.25



Distribution of High Risk HPV 16/18 in different age groups

Fig. 1. Distribution of HPV 16/18 in the different age groups

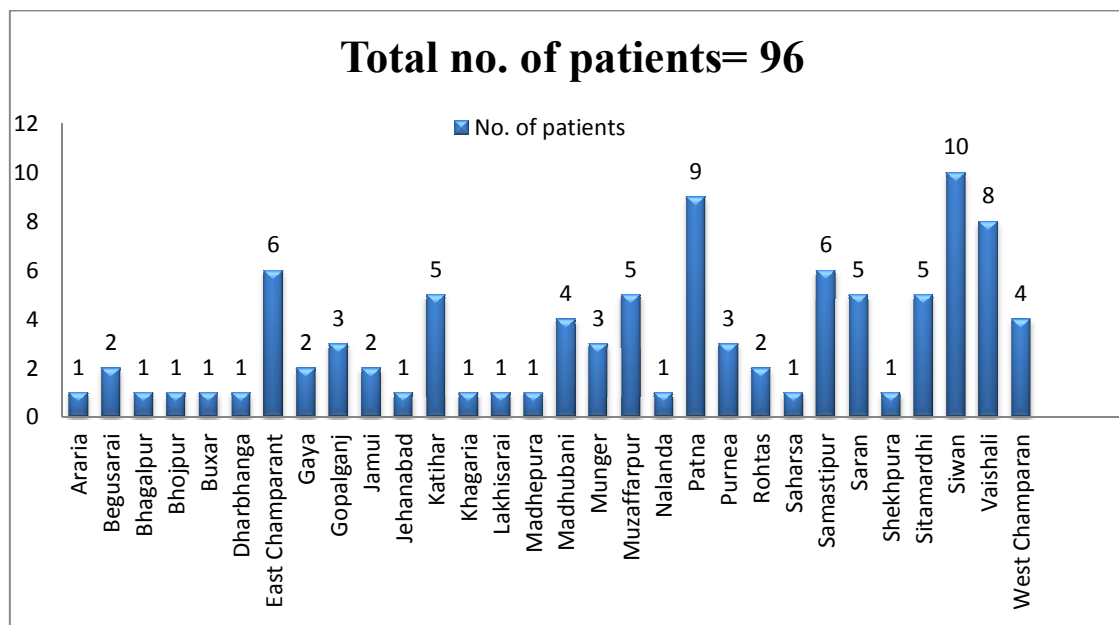


Fig. 2. Represent the patients included are from different districts of Bihar

Table 3. The comparative HPV distribution in different zones of India

Place	Study design	Method	Prevalence (%)		References
			HPV 16	HPV 18	
North India					
UP	N=106	PCR assay using a reverse line blot hybridization assay	HPV-16 73.6	HPV 18 14.2	Bhatla et al. [32]
	N=40	Southern blot hybridization and in-situ hybridization (isotopic)	HPV-16 55	HPV 18 35	Seth P et al.[33]
	N=96	Southern blot hybridization; PCR	HPV-16 64	HPV-18 3	Das BC, et al.[34]
Haryana	N = 110	PCR	HPV 16 84.11	HPV 18 72.89	Kaidan et al. [35]
Central India					
Madhya Pradesh	N=270	PCR	HPV-16 73.7	HPV-18 11.9	Munjaj et al.[36]
Chhattisgarh	N= 185	PCR	HPV 16 63.9	HPV 18 11.1	Negi et al.[37]
South India					
Tamilnadu	N=205	PCR (GP5+/6+); typing by ELISAbased	HPV-16 61	HPV-18 14	Franceschi et al.[38]
	N=119	line blot assay	HPV-16 60.5	HPV-18 14	Peedicayil et al.[39]
	N=43	DNA amplification with MY09/MY11	HPV-16 53	HPV 18 13	Munagala et al.[40]
	N=28	Line Blot Assay	HPV-16 78.6	-	Peedicayil et al. [41]
	N=110	PCR	HPV-16 69.1	-	Nair et al. [42]
Telangana	N=41	PCR Based Line Blot assay	HPV-16 66.7	HPV 18 19.4	Sowjanya et al. [31]
Karnataka	N= 60	PCR	HPV 16 89.7	HPV 18 86.2	Kulkarni et al.[43]
West India					
Maharashtra	N=72	In-situ hybridization (non-radioisotopic)	HPV-16 44	HPV-18 54	Menon et al.[44]
	N=180	PCR	HPV-16 81.7	-	Gheitet al.[45]
Gujarat	N= 52	PCR	HPV 16 90.3	HPV 18 9.7	Patel et al. [46]
North East					
Assam	N = 54	PCR	HPV 16 95.83	HPV 18 4.16	Das et al. [47]
East India					
West Bengal	N=117	PCR (consensus); hybridization	HPV-16 63	HPV-18 33	Chatterjee et al.[48]
Orissa	N=71	L-1-specific PCR and Southern hybridization	HPV-16 40.9	HPV 18 8.2	Nagpal et al. [49]
Bihar	N= 96	RT- PCR	HPV 16 77.08	HPV 18 16.67	Present Study

In present study, HPV infection was found very high (100%) with HPV 16 and HPV 18 as the prominent infection (Table 2). The concordant result was also found in a study conducted in Chennai, Tamil Nadu where the reported prevalence was 99.4% in cervical cancer biopsy samples [27]. The study used real time PCR technology for the genotypic identification of HPV. The technique used was of high sensitivity and specificity which might be the reason for 100% prevalence of HPV 16/18 in present study. However, a study from Andhra Pradesh, India reported 66.7% and 19.4% prevalence of HPV 16 and 18 respectively[28]. The reported prevalence of HPV 16/18 in the study is in close proximity to a recent study with large sample size reported in India and is also similar to the prevalent strains of HPV found in South-East Asia [22-24,26,27]. Therefore, we may expect that a prophylactic vaccine focussing HPV 16 could curtail a major pie of the cervical cancer burden in Bihar, along with nearby states of Jharkhand, West Bengal and Uttar Pradesh.

HPV vaccines namely Cervarix and Gardasil are available in the market. The vaccine has huge potential for developing nations like India where gynaecological problems are posing great concern. Vaccination would not only reduce cancer mortality but also curtail the screening cost to the nation which is concern in a resource limited setting like India especially Bihar, the state which faces both flood and drought every year. Even today, there exists feasibility for Human Papilloma Virus vaccine trial in India under the flagship of the Indian Council of Medical Research(ICMR),India [29]. It is of utmost importance to know the prevalence of the most frequent high risk HPV types in different geographical distributions of India. This became the basis of our study so that we can formulate a cost effective central vaccination strategy for mitigation of this gynaecological malignancy. Exhaustive and inclusive HPV 16/18 type analysis of cervical cancer biopsy from the South, East, West, North and North-eastern states of India is needed to augment a nationwide vaccine strategy for India. Studies carried out in different zones of India have been summarized in Table 3. Moreover, an initiative by different state government has been taken. New Delhi initiated free HPV vaccination on 7th Nov 2016 followed by Punjab on 23rd Nov 2016. Though we don't have exact figures about cervical malignancy burden in Bihar but as per patient registry data of MCSRI, Patna 2016, the annual registered new patients from all cancers is 20,746 and cervical

cancer alone constitute 14% (2,904) of the patients [30]. This data is a clear indicative of alarming incidence of cervical cancer in Bihar. Bihar as a state too should take an initiative in this direction considering the heavy cervical cancer incidence and poverty in the state of Bihar, India. Scientifically and rationally justified HPV 16 vaccination can help reduce the Cervical Cancer incidence and mortality in Bihar, India [31]. This is the pilot study of HPV 16/18 distribution in cervical cancer in Bihar.

5. CONCLUSION

The study depicts the prevalence and pattern of HPV 16/18 in the state of Bihar .The study results are in close proximity with studies establishing the link of HPV16/18 as a major risk of cervical malignancy occurrence. HPV types16/18 distribution in Bihar, India is equivalent or more than to those reported from the studies carried out in different regions of India. The high prevalence of high risk HPV-16 in cervical cancer tissues suggest that the compelling vaccination strategy against HPV 16 in adolescence girls might considerably reduce the cervical malignancy in the state of Bihar, India. HPV 18 prevalence is less in Bihar as compared to other parts of India. Regional data from different states of India are more important than developing strategies on the basis of pooled data from other countries. HPV 16/18 vaccination will surely curtail the incidence of cervical cancer in Bihar, India.

6. STRENGTH AND LIMITATIONS OF THE STUDY

This study is the pioneer study for the prevalence and genotypic pattern of high risk HPV 16/18 in the state of Bihar .We report 100 % prevalence of HPV 16/18 in the biopsy specimens of cervical cancer .This would give a notion to the health department, Bihar for strategic planning for HPV vaccination as New Delhi and Punjab has taken initiatives in this regard by adopting HPV vaccination in 2016.

HPV genotyping should have been done in more diversified sample from different districts and on more samples. Prevalence of HPV in normal population of Bihar has not been done

CONSENT AND ETHICAL APPROVAL

The Ethics Committee of MCSRI, Patna approved the study (MCS/Admin/2017-18/2012).

Written Informed consent was obtained from patients and the objective and details of the study was explained.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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