Prevalence of human papilloma virus and their high-risk genotypes in Sri Lankan women

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Abstract Human papilloma virus (HPV) causes cervical cancer in women and approximately 700 deaths have been reported annually in Sri Lanka due to this cancer. Despite, attempts have not been made to investigate the prevalence of HPV amongst Sri Lankan women with normal cytology. In this study, a polymerase chain reaction based assay was set up to detect HPV in both normal and abnormal cytology and the positive samples were then tested for the genotypes, HPV 16 and HPV 18 as they have been identified as the high-risk types associating with cervical cancer. Eighty-four (number = 84) clinical samples (age range 27–69) analyzed in this study indicated that the prevalence of HPV, regardless of cytological abnormalities was 15.5%, ($n = 13$, 95% class interval ± 7.7) while it was 100% ($n = 3$) for those with abnormal cytology. Association of HPV 16 and HPV 18 among the abnormal cytology was 0 and 50% ($n = 1$), respectively and further, the prevalence of HPV 16 and HPV 18 in women was found to be 3.6% ($n = 3$, 95% CI ± 4.0) and 2.4% ($n = 2$, 95% CI ± 3.3), respectively. Moreover, age wise prevalence analysis revealed women of the age of 35-years or more to have higher HPV prevalence. The prevalence of HPV among normal cytology is 12.3% ($n = 10$, 95% CI ± 7.2) which is similar to the rates in other regions of Asia (China 15.4%; India 10.43%). Finally, higher prevalence of HPV in women of the age of 35-years

or more in Sri Lanka, especially with malignant types call for such age group to be screened for proper clinical intervention to be made in reducing the incident of cervical cancers. This is the first report of prevalence of HPV among women with normal cytology in Sri Lanka.

Keywords Human papilloma virus (HPV) · Cervical cancer · Prevalence · Cytology

Introduction

Papillomaviruses (“Family *Papillomaviridae*”) cause a variety of clinical manifestations such as warts, lesions and severe form of cancers in humans, depending on the types of infection [4, 16]. Although several types of genital human papilloma virus (“the genus *Alpha*”) contribute for the development of cervical cancers, some types are more virulent in the process. They are referred to as the high-risk types and include HPV 16 and HPV 18 [8].

Cervical cancer that arises in the transformation zone of the uterine cervix is the second most common cancer among women in Sri Lanka as well as in the World [12]. It has been shown that there is a strong association of HPV with cervical cancer, more than 99% worldwide and around 90% in Asia [5]. In 2012, it was estimated that around 1700 new cervical cancer cases were present in Sri Lanka, with roughly 700 annual deaths while 530,000 new cervical cancer cases were reported worldwide with 275,000 annual deaths [11]. The prevalence of HPV 16 and HPV 18 is the highest and around 83% of the total cervical cancer cases reported in Sri Lanka [5, 19]. However, no research has been conducted to evaluate HPV 16 and 18 infections among the Sri Lankan women with normal cytology. HPV is regarded as an ubiquitous infection agent among the general population [21, 22] and

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due to anatomical differences, cervix more frequently harbors HPV than male genital tissues [20]. Further, the incidence of HPV infections and cervical cancers vary significantly with the age of women [7, 17] and women between 20 to 25 years have the highest HPV prevalence (approximately 20% of women) [7] and women close to the age of 55 years show the highest cervical cancer incidence compared to other age groups [22]. Furthermore, women more than 55 years of age have shown an increase in HPV infections again, due to weakening of immune activity [22]. In Asia, regardless of any cytological abnormality and age, the prevalence of HPV is around 12%, out of which 4% is due to the infection of HPV 16 and 18 [13].

After persistent infection is caused by HPV on the cervical tissues, infected tissues show an unnatural morphology including cervical lesions and abnormal cervical cytology which are key indicators for cytological assessment of HPV [3]. Currently in Sri Lanka, the evaluation of cervical cancers is carried out by cytological assessment. However, many previous research has shown that HPV can be present without any clinical symptoms [3, 6, 7], which questions the reliability of such assessment in determining HPV infections [15]. Moreover, HPV infections are transient inside the cervical cells; therefore, these initial infections may get wiped out by immune action without any cervical abnormality [15]. Further, due to the inability to grow HPV under in vitro conditions [7, 14, 18], their identification cannot be relied on methods other than molecular biological techniques. Therefore, the objective of this study is to use a PCR-based molecular diagnostic assay set up to evaluate the prevalence of human papillomavirus and their high-risk genotypes (HPV16 and 18) in women of Sri Lanka with or without cervical alterations.

Materials and methods

Sample collection and storage

This research was conducted between May to August 2016. Eighty-four ($n = 84$) Sri Lankan women who visited to the Durdans Hospital, Colombo, Sri Lanka were screened for the presence of HPV and their cytological abnormalities were recorded upon obtaining their written consent. Surplus samples collected for laboratory diagnosis at the Durdans Hospital were blinded, coded and used in the study. No patient identifiable data were used in the study. Phosphate buffered saline (pH 7.0) mixed with Tetracycline hydrochloride (Himedia) [2] for the sample preservation (10 mg/mL) was used as the sample collection media. Samples collected using a cervical brush by an experienced medical doctor were immediately dipped in the collection buffer and stored at 4 °C until further processing.

DNA extraction

To recover cervical cells in the form of a cell pellet, tubes containing cervical brush was shaken vigorously for 5 min and centrifuged at 7000 g for 10 min. The DNA of cell pellet was then extracted using GenoSpin GTM genomic DNA extraction kit (CeyGen Biotech, Sri Lanka) per manufacturer's instructions. The cell pellet treated with 200 µL of tissue lysis buffer and 40 µL of Proteinase K (20 mg/mL) was incubated at 56 °C for 30 min and 400 µL of binding buffer and 300 µL of absolute ethanol were added to this. Resulting mixture was then placed on a GenoSpin RTM column and centrifuged at 10 000 g for 1 min, and the column was washed with 500 µL of each Wash buffer 1 (WB1) and Wash buffer 2 (WB2), by centrifuging at 10,000 g for 1 min. Then the solution was eluted with 20 µL of Elution buffer to recover DNA, which was stored at − 20 °C until used.

PCR amplification of the viral DNA

To amplify the variable region (part of the L 1 region of HPV genome) flanked by two conserved sequences of the HPV genome, PCR reaction were performed in 25 µL containing 1 µM of GP5+ & GP6+ primer pair [10] (GenScript, USA), 0.2 mM of dNTP, 1.5 mM MgCl₂, 1X ThermoReadTM Taq DNA polymerase PCR buffer, 1 units ThermoReadTM Taq DNA polymerase (CeyGen Biotech, Sri Lanka), and 1 µL of eluted DNA. PCR cyclic parameters for GP5+ & GP6+ primer pair were initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 45 °C for 30 s, primer extension at 72 °C for 30 s and final extension at 72 °C for 6 min. Samples that were recorded positive for GP5+ & GP6+ primer pair were subjected to amplification with H16 (5'-ATTATGTGCTGCCATATCTACTTCAGAA-3') and H18 primer (5'-TGCTTCTACACAGTCTCCTGTACCTGGGCA-3') specific to HPV genotype 16 and 18 [1]. PCR conditions and cyclic parameters were the same as GP5+ & GP6+ primer pair amplification. DNA from HeLa cells was used as the positive control and human blood cells were used as the negative control. The PCR products were run in 2% agarose gel, and visualized under UV-light.

Results and discussion

Prevalence of HPV

In this research two types of data were collected i.e., the presence/absence of HPV infection in the sample collected and their clinical information. Statistical analysis of data obtained with regards to presence/absence of HPV

infection is given in the Table 1. The survey revealed that the prevalence of HPV among Sri Lankan women with normal cytology is approximately similar to the other regional countries. Furthermore, the data consisted of two main features, one is the higher prevalence of HPV infection among women at 35 years of age and the second is the accumulation of high-risk types among women, especially over 55 years of age.

Among the 84 random samples, thirteen samples 15.5% ($n = 13$, 95% class interval ± 7.7) were positive for HPV (age range was 27–69 years) regardless of any cytological condition (overall prevalence). HPV prevalence was 12.3% ($n = 10$, 95% CI ± 7.2) in eighty-one samples with normal cytology and this value is similar to the values reported in other regional countries such as China 15.4% and India 10.43% [13]. Moreover, HPV prevalence for abnormal cytology was 100%; that is samples positive for lesion was also positive for HPV (Table 2). Further, the sensitivity of the molecular diagnostic assay can be calculated as number of samples showing the HPV infections over the number of samples showing the detectable cervical abnormalities. Here, the total numbers of HPV infections are thirteen (13) and only three (3) samples showed the cervical abnormalities. Therefore, the sensitivity of the molecular diagnosis was 4.3 times higher than the cytological assessment.

Prevalence of genotypes HPV 16 and HPV 18

Positive samples from general HPV-PCR were subjected to the genotype-specific PCR and in this assay, three samples, 3.6% ($n = 3$, 95% CI ± 4.0) and two samples, 2.4% ($n = 2$, 95% CI ± 3.3) were positive for the HPV 16 and HPV 18, respectively (Table 1). HPV 16 has been reported as the commonest type among the HPV infections [8]. However, in this study among the HPV positive samples, only in three samples 23% ($n = 3$) having HPV 16 infection were detected.

HPV infection retards terminal differentiation of epithelial tissue of cervix making this tissue to be more prone to cervical lesions, which is the immediate clinical manifestation of the HPV infections [11]. Such observation was made in the current study in the sample that was infected with HPV 18 ($n = 1$, 50%), but not with the three samples that were infected with HPV 16 (Table 2). Further, ten samples ($n = 10$, 77%) did not show any clinical abnormality and they were symptomless. HPV infections are generally considered to be multiple in nature [9] and among samples detected to be positive for HPV in this study, one sample (7.7%) was positive for both HPV 16 and HPV 18 (Table 2). Since only two genotypes could be detected by this assay, determination of actual co-infections by other genotypes of HPV was not possible. Four samples, 4.8% ($n = 4$, 95% CI ± 4.5) were positive for

Table 1 Age-wise prevalence of HPV among Sri Lankan women

Sample size (n)	Age group	Median	HPV+ (%) [CI 95%]	HPV16+ (%) CI 95%]	HPV18+ (%) [CI 95%]
4	21–30	25.5	–	–	–
17	31–40	35.5	3 (17.6%) [CI ± 18.1]	1 (5.9%) [CI ± 11.21]	–
23	41–50	45.5	3 (13.0%) [CI ± 13.7]	–	–
27	51–60	55.5	4 (14.8%) [CI ± 13.4]	–	1 (3.7%) [CI ± 7.1]
13	61–70	65.5	3 (23.1%) [CI ± 22.9]	2 (15.4%) [CI ± 19.6]	1 (7.7%) [CI ± 14.5]
84	Total		13 (15.5%) [CI ± 7.7]	3 (3.6%) [CI ± 4.0]	2 (2.4%) [CI ± 3.3]

Table 2 Clinical manifestations due to HPV infections

Condition	Number of samples	Cervical lesions (%)	Normal cytology (%)
HPV negative	71	0	71 (100%)
HPV positive	13	3 (23.1%)	10 (76.9%)
HPV+ and HPV 16+	3	0	3 (100%)
HPV+ and HPV 18+	2	1 (50%)	1 (50%)
	1	0	1 (100%)

either HPV 16 or HPV 18 among total samples and they were considered as the most virulent and cancer causative types. However, no cervical cancers were detected in individuals from whom these samples were collected.

Age-wise prevalence of HPV

HPV among five age classes ranging from 26 to 69 years in Sri Lankan women was analyzed to study age-wise prevalence (Table 1) and in doing so, median of the age class was used to represent the group. In this analysis, no HPV positive samples in the first group, 21–30 (Median 25.5, N = 4); three HPV positive samples, 17.6% (95% CI $\pm$ 18.1) in the second group, 31–40 (Median 35.5, N = 17); three HPV positive samples, 13.0% (95% CI $\pm$ 13.7) in the third group, 41–50 (Median 45.5, N = 23); four HPV positive samples, 14.8% (95% CI $\pm$ 13.4) in the fourth group, 51–60 (Median 55.5, N = 27) and three HPV positive samples, 23.1% (95% CI $\pm$ 22.9) in the final group, 61–70 (Median 65.5, N = 13) were found.

Using above data, a histogram (Fig. 1) was generated to evaluate the HPV prevalence with the age and accordingly, the highest HPV prevalence was observed at latter stages (after 50 years). Especially HPV 16 and 18 genotypes are more observable after 50 years of age (Table 1). This observation is compatible with high frequency of cervical cancers after 50 years of age. In this analysis, a local peak was observed at roughly 35 years of age, supporting the recommendation made by Sri Lankan Medical Council that a female of 35-year of age to undergo pap test.

In conclusion, it was found in the current research that HPV infections for women with normal cytology in Sri Lanka were similar to those values determined for India

and China suggesting that HPV infections show similarities in regional countries with a uniform distribution, yet again confirming the characteristic nature of an ubiquitous pathogen. Further, outcomes of this research suggest that women at the age of 35-year or more have higher chances of HPV infection, women more than 55 years have higher prevalence of malignant genotypes such as HPV 16 and HPV 18, strengthening previous observation that, higher incidence of cervical cancer is seen in older women.

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Fig. 1 Incidence of HPV infections regardless of cytological abnormalities within median age of age-groups (Table 1). Solid line shows the HPV prevalence. This graph is characterized with two features, first one is the local peak roughly at 35 years of age and the second one is the variation of the infection with age

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