Detection and genotyping of human papillomavirus in patients with oral and oropharyngeal carcinomas at Cancer Institute, Maharagama

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Statement of Originality

I hereby declare that the work presented here in the dissertation is my own and that no part of the dissertation has been submitted earlier or concurrently for any other degree.

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CONTENTS

LIST OF TABLES

LIST OF ANNEXURES

LIST OF ABBREVIATIONS

CDK	-	Cyclin dependent kinase
CI	-	Confidence Interval
DNA	-	Deoxyribonucleic acid
E2F	-	E2 factor
EPI	-	Expanded Programme on Immunization
FDA	-	Food and drug authority
GM-CSF	-	Granulocyte-Macrophage Colony stimulating factor
IARC	-	International Agency for Research on Cancer
mRNA	-	Messenger ribonucleic acid
n	-	Number
OPD	-	Out Patient Department
OR	-	Odds ratio
PCR	-	Polymerase chain reaction
p53	-	Tumour protein 53
STI	-	Sexually transmitted infection

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ABSTRACT

Introduction

Human Papillomavirus (HPV) causes oral and oropharyngeal carcinomas (OC and OPC) worldwide predominantly by type 16. This group has distinct profile of clinical presentation, diagnosis, management, prevention with good prognosis compared to HPV unassociated cancers. In spite of rising incidence of HPV associated OC and OPC globally, data on HPV in OC and OPC is very limited in Sri Lanka.

This study aims to detect and genotype the HPV in OC and OPC patients and to describe associated factors of HPV infection in OC and OPC patients at National Cancer Institute, Maharagama.

Methods

This comparative cross sectional study was carried out for four months from 15th of December 2015 to 15th of April 2016. Swabs from lesions were collected from 127 of OC and OPC patients from Cancer Institute. Swabs were collected from 127 of age and sex matched non-cancer patients from Out Patient Department, National Hospital. HPV was detected and genotyped using commercially validated genotyping real time polymerase chain reaction kit including 12 high risk HPV genotypes. Interviewer based questionnaire and clinic records were used to collect sociodemographic and other data from both groups. Statistical analysis was performed using SPSS with descriptive statistics and multivariate logistic regression models.

Results

Males were predominant (95.3%) and the highest proportion (44.1%) was of age 51-60 years in both cancer and non-cancer groups. Fifteen percent (19/127) of OC and OPC group was infected with HPV types 16, 39, 52, 58, 59 with predominance of type 16. Of non-cancer group, 3.1% (4/127) was infected with HPV 52 and 59 without detection of type 16 or 18. Low education level, poor dental hygiene, smoking, betel chewing, large

tumour size, presence of lymph nodes, primary tumour without recurrence and OPC type showed significant association (p value≤0.05) with HPV in cancer group.

Conclusion

Human papillomavirus was detected in 15% of OC and OPC patients with predominant type of 16 and 3.1% in non-cancer group without type 16. Human papillomavirus positivity was significantly associated with OC and OPC patients. Multiple socio demographic, behavioral and tumor factors were associated with HPV infection in OC and OPC group.

CHAPTER 1

INTRODUCTION

Human Papillomaviruses (HPV) belongs to *Papillomavirus* genus of the *Papillomaviridae* family. They are non enveloped, small and epitheliotropic DNA viruses. So far, more than 200 types of HPV have been identified. HPV is divided in to high-risk (HR) and low-risk (LR) types according to the ability of virus to transform epithelial cells to malignant cells. The LR types like 6 and 11 are associated with benign lesions like warts, while HR types like 16 and 18 progress to malignant lesions. The E6 and E7 of HR-HPV types demonstrate transforming properties via binding of various cellular factors and tumor suppressor proteins such as p 53 gene product and retinoblastoma proteins (Bonnez, 2015).

Head and neck carcinoma (HNC) is the sixth commonest type of cancer group globally and eighth commonest cause for cancer death. Carcinomas of the oral cavity (OC) and oropharynx (OPC) are the most common type of HNC according to Global Cancer Statistical data in International Agency for Research in Cancer (2017). Cigarette smoking, betel chewing and alcohol are the main causes of these cancers. However, part of the population develops OC and OPC without having been exposed to these risk factors suggesting other causes such as viral agents, most specifically sexually transmitting HPV. Molecular epidemiological studies have shown a strong correlation between oncogenic HPV infections especially types 16 and 18 and a subset of oral and oropharyngeal pre cancer and cancers (Jayaprakash *et al*, 2011).

HPV positive OC and OPC have been recognized in the past 10 years as a distinct disease entity. Clinically, HPV positive cancers are present in younger patients mostly who have less exposure to tobacco and alcohol and commonly affect the base of the tongue/ tonsils. HPV related Squamous Cell Carcinomas (SCC) have characteristic histological appearance of poorly differentiated, non-keratinized with a basaloid appearance. These

cancers are distinct from other Head and Neck SCC (HNSCC), and present in an advanced stage with high nodal category.

But this has improved survival and prognosis, higher than 30%, irrespective of treatment modality along with fewer rates of disease relapse with improved survival. These patients have less chance to develop 2nd malignancies in contrast to HPV negative patients (Fakhry *et al*, 2008).

De-intensification of therapy in this group with good prognosis to reduce the toxicity is a major clinical relevance and is currently being tested in clinical trials. It includes decreasing the dose of radiation (de-escalation) or change to targeted therapy with Cetuximab instead of Cisplatin based therapy. Immunotherapy targeting various immune mechanisms is currently a major rapidly changing area of cancer management.

HPV associated HNSCC are also preventable by vaccines causing specific immune responses against HPV theoretically and studies are still going on to determine the efficacy of the available vaccines to prevent these cancers in addition to cervical cancers(Chi *et al*, 2015). And there is evidence that HPV vaccination have effect on oral HPV infection in addition to prevention of anogenital HPV infection (Hirth et al, 2017).

A new model on risk stratification was generated considering the HPV status predicting 3 year overall survivals rather than traditional TNM (Tumour, Nodes and Metastases based) staging, because survival according to TNM status was not correlated with survival of patients having HPV positive disease. There is an interest in finding the novel biomarkers for patients at risk of recurrence such as E7 antibody clearance, p16 and persistence of HPV DNA in oral rinse(Swiecicki *et al*, 2016).

Although a lot of studies and interest has been developed on this HPV positive tumor category worldwide, very limited studies and data are available for Sri Lankan population. In other Asian countries prevalence rates for OPC and OC are 46.3% and 33% respectively and comparatively rates are high than the rest of the world (Kreimer *et al,* 2005). In Sri Lanka, lip, oral cavity and pharyngeal cancer group is in top of the cancer incidence in male population with increasing age specific rates. It is the second

commonest in females and third place when considering both sexes according to Sri Lankan Cancer Incidence Data of cancer registry (2010). In 2017, HPV vaccination was introduced to EPI schedule for girls of 10-11 years of age in Sri Lanka according to Ministry of Health, Sri Lanka (2017).

Over the past two decades there is a significant rise in the number of new HPV associated cancers, increasing from 16.3% to 71.7%, with HPV 16 as the predominant type. And it is accompanied by a 50% decline in the incidence of HPV negative OPC globally (Swiecicki *et al*, 2016). Global data on HPV prevalence may not compatible with Sri Lanka, because cultural and other behavioral factors such as smoking, betel chewing and sexual behavior of Sri Lankan population are different from Western population.

Therefore, it is important to find out the prevalence, genotype distribution and associated factors of HPV infection in OC and OPC patients of Sri Lanka to develop own strategies to manage and prevent these types of cancers.

The objectives of the study were as follows:

General Objective

To detect and genotype identification (genotypes 16 and 18) of HPV in oral and oropharyngeal carcinomas in patients attending Cancer Institute, Maharagama

Specific Objectives

1. To determine the prevalence of HPV among oral and oropharyngeal carcinomas in patients attending Cancer Institute, Maharagama
2. To determine the HPV genotypes (genotypes 16 and 18) and its distribution among oral and oropharyngeal carcinoma patients in the study population
3. To describe socio-demographic, behavioral and other factors associated with HPV infection among oral and oropharyngeal carcinoma patients in the study population

CHAPTER 2

LITERATURE REVIEW

2.1 History

Papillomaviruses are pathogens of an ancient family causing infections of epithelial tissues of higher vertebrates like mammals. The name "papilloma" is a Latin term with a meaning of *papilla* for nipple and *oma* for tumour. They produce benign skin tumours/papillomas, containing infectious viral particles (McCance, 2009). HPV is a group of closely related papillomavirus strains that can infect humans(Burd, 2016).

They have species specificity and HPV can infect only humans. But there are rare cases of crossing infections among species, ex- HVP DNA isolated from cutaneous lesions of the cat. They are tissue tropic, with a complete life cycle in a fully differentiated squamous epithelium (Dutra *et al*, 2012). The lesions have been identified from fifth century BC but the virus was first visualized in the 1950s soon after electron microscopy came into use (McCance, 2009). Hand and plantar warts are the most common skin manifestations caused by this virus in humans. Twenty five years back, the first Papillomavirus was isolated from a cervical carcinoma and it was mentioned by Durst *et al* (1983). In 1995, International Agency for Research on Cancer (IARC) recognized that HR-HPV 16 and 18 can cause cancers of humans. The World Health Organization (WHO) ascertained etiological association of HPV with HNSCC in 2007 according to IARC report (2007).

2.2 Virology

Papillomaviruses belong to the family *Papillomaviridae*. Papillomaviruses have 18 different genera. Human Papillomavirus belongs to 5 genera called Alpha, Beta, Gamma, Mu and Nu. Of these, alpha genus has the viruses causing mucosal tumours in humans, and viruses of beta genus are associated with the development of cutaneous tumours. There are particular features characterizing each genus such as beta and gamma papillomaviruses lack an E5 ORF (open reading frames). A genus is further divided into species defined by a particular representative etc. HPV 16, an alpha papillomavirus is the

representative type of species 9, which also includes types 31, 33, 35, 52 and 67 (Bonnez,2015).

HPV still have not been grown in vitro using cell culture techniques, this may be due to the fact that the replicative cycle of the virus occurs in squamous epithelium with the full cycle occurring only in the most differentiated cell layers. Therefore the existence of a large number of distinct HPVs became evident only with the development of recombinant DNA technology. Well over 200 HPV types have been identified and sequenced from lesions, according to the genomic sequence of L1, which is encoding the major capsid protein (Doorbar *et al*, 2012). Nucleotide sequences of genotypes differ each other by more than 10%. Genotypes with 80–90% similarity are grouped in same species, and they share important features like tissue tropism, pathogenicity and disease manifestation (Doorbar *et al*, 2012).

HPV can be categorized into two broader groups according to the site of infection, cutaneous HPV which infect keratinized squamous epithelium and mucosal types that infect non keratinized squamous epithelium. Mucosotropic viruses are classified into low-risk (LR) or non-cancerous types like HPV 6 and 11, and potentially high-risk (HR) cancerous types including HPV 16, 18, 51, and 53 according to carcinogenicity. The IARC currently defines 12 HR- HPV types cause malignancies in humans. They include types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59 (States *et al*, 2017; Doorbar *et al*, 2012).

Papillomaviruses are non enveloped small viruses of 55 nm in diameter with an icosahedral capsid. Virion particles have at least two capsid proteins called L1-minor structural protein and L2-major structural protein and L2 constitutes the 80% of the weight of virion.

HPV is a double stranded DNA virus.HPV genome consists of about 7900 base pairs and all coding sequences (open reading frames/ORFs) are arranged in single DNA strand. All Papillomaviruses share the same genomic organization. The genome is divided into an E/Early region (contains genes E1-E2, E4-E7), L/Late region (contains L1, L2), and an URR/ Upstream Regulatory Region. E1–E7 has functions in regulating and promoting

HPV DNA replication. L1 and L2 are transcribed in productively infected cells only and code for major and minor capsid proteins. These major and minor proteins are required for assembly of virions, accumulation and release into the environment while non coding URR involve in controlling DNA replication and transcription of early and late regions (Bonnez, 2017).

2.3 Life cycle

The natural host cells to complete the infection cycle are the squamous epithelial cells, which is mucosal epithelium of body openings or external cutaneous skin. Most HPV types have tropism for either cutaneous or mucosal tissue, but some genotypes can infect and multiply in both. HPV can infect the squamous epithelium when basal or parabasal cell layers are exposed by wounding. The basal layer is essential for viral infection and is the only cell within epithelium capable for establishing the infection (Miller *et al*, 2012).

The proximal epithelial lining of a body opening is stratified squamous, with the more internal epithelium typically consists of columnar epithelium, some of which have cilia, like in the respiratory system. A band of dividing keratinocytes called as transformation zone leads to a squamous-columnar junction of the body opening. They are located in larynx, sinuses, urethra, cervical region, and anorectal junction. They are particularly susceptible to HPV infection and also support reproduction.

Oropharynx is more susceptible to HPV transformation in gastrointestinal tract due to its similar features with the cervix like easy access and the same embryonic development. Also tonsils contain deep invaginations of the mucosa and favor the taken up and processing of antigens facilitating the viral entry into basal cell layers.

A complicated and still poorly defined process is required for attachment to the host cells, internalization and intracellular trafficking. Affinity of HPV towards basal layers and not to differentiated cells is owned due to preferential binding with basement membrane proteins like Heparan Sulfate and glycosaminoglycans specific to the basal layers (Miller *et al*, 2012). This binding will be followed by a binding with a second receptor, possibly laminin which leads to entry of the virus through clarithrin coated pit mechanism into an

endosome. The second trafficking pathway will be the Golgi apparatus, where a membrane protease cleave the L2 and release HPV DNA.

It initiates the replication as an episomal DNA in basal layers with generation of low copy numbers (< 20-50/cell). Replication of the virus depends on replicative machinery of the cell and in part under non-cancer by transport of E1 within the nucleus. Histones associated with viral DNA displaced and the supercoiled conformation is unwound with the beginning of HPV DNA replication. E1 and E2 play a major part in viral DNA replication.

Synthesized viral DNA is subsequently encapsidated in a process which is associated with cellular histone proteins. The L2 transiently binds with E2 protein and guides the DNA into the aggregation of viral L1 and L2 which finally forms capsid.

Virus particles are released by disintegration of upper squamous epithelium which may be facilitated be E1-E4 proteins. These desquamating cells are considered as infectious (Bonnez,2017).

2.4 Transmission

HPV infection is mainly a sexually transmitted infection including oral sex. In addition, HPV can also be transmitted through close personal contacts via infected genital skin, mucous membranes or body fluids.

2.5 Epidemiology

Prevalence of HPV associated HNSCC

In the last decade there was evidence that HPV is a cause for a subgroup of HNSCC in addition to anogenital cancers. Jayaprakash (2011) reported in a meta- analysis of "quantification of the prevalence of HPV types 16 and 18 in oral cavity and oropharyngeal dysplasia lesions", that HPV 16/18 infections occur in the oral cavity during the early phase in carcinogenesis.

According to the IARC summary report (2017), IARC has decided that, (a) there is adequate evidence for the carcinogenicity of HPV 16 within the oral cavity, oropharynx and (b) only limited evidence for cancers of larynx.

HNSCC contributes for 3.5% of all malignant tumours in America and Europe , but in the rest of the world, such as Southeast Asia and Brazil, they are more common, being the 6th commonest carcinoma worldwide(Jemal *et al*, 2011).

The epidemiology of HNSCC has changed rapidly in past two decades. With the reduction of tobacco use, incidence of the tobacco associated HNSCC has decreased, but the incidence of HPV positive OPC is increasing in the developed world (Chaturvedi *et al*, 2011).

Estimated prevalence of HPV infection in normal oral mucosa, pre-cancerous lesions, and OSCC are variable, and comprehensive studies have reported results that ranged from 0%- 100%. These results depend on the method of sampling, detection methods used , and the site of tumours(Akhter *et al*, 2013).

HPV is positive in about 25% of all HNSCC, and the most of these HNSCC are OPSCC affecting tonsils and base of the tongue (Kreimer *et al*, 2005).

In addition to the 492,800 of carcinoma cervix due to HPV worldwide each year, it also causes about 30,000 OPC. At present, HPV is the main cause for OPC in developed world, in 45–90% of patients (D'Souza *et al*, 2007; Kreimer *et al*, 2005).

HPV has also been detected in a small subset of laryngeal (24%) and OC (23%) cancers (Kreimer et al, 2005). According to the current data, HPV associated OC and OPC may surpass smoking related OC and OPC and also the cervical cancer by 2020 (Chaturvedi *et al*, 2011).

The incidence rate of OC and OPC is rising in Asia with changes in a number of factors including site of occurrence, male to female ratio, age and occurrence in people with no known risk habits (Rao *et al*, 2013).

In Asia, prevalence rates for HPV associated oropharyngeal and oral carcinomas are 46.3% and 33% respectively, and comparatively rates are higher than in rest of the world (Kreimer *et al*, 2005). These higher rates when compared to worldwide may due to higher number of incidence of above carcinomas in Asian countries. A study in the Osaka University Hospital, Japan showed a high rate of HPV related OC in Asia. HPV positivity was 34.4% in 163 Japanese OPC patients (Maruyama *et al*, 2014).

Akthar (2013) had done a study to describe the association of HPV in Bangladeshi OC patients and find out the crude prevalence as 3%.

Few studies have been done in India and they showed range of HPV prevalence in HNSCC from 17% to 73%. This discrepancy may be due to limited number of participants and various sensitivities of HPV detection laboratory methods in each study (Balram *et al*, 1995; D'Costa *et al*, 1998; Nagpal *et al*, 2002).

In Sri Lanka, oral and oropharyngeal carcinomas are the second most prevalent group of malignancy and most prevalent type among males (22% of all cancers in males and 26% among in females) according to cancer registry, Sri Lanka (2010).

Although numerous studies have confirmed the importance of HPV associated oral and oropharyngeal cancers worldwide including neighborhood India, current status of the disease in Sri Lanka is questionable. Is it similar to the developed American and European countries where HPV related oral cancer is currently one of the major disease burdens for the health care systems? Or is the condition same as in India?

In Sri Lanka very limited data is available on HPV infection in oral and oropharyngeal carcinomas. There are no published data available on HPV infection associated oral and oropharyngeal malignancies in Sri Lanka in the ICO Information centre on HPV and Cancer, 2014.

Jayasooriya *et al* (2003) did a study to determine the prevalence of HPV and correlate the HPV status with clinical and pathological parameters of OC patients from Sri Lanka. They used formalin fixed biopsies from OC patients and detected HPV by PCR. The HPV prevalence of the overall sample was 37.2% and HR-HPV types, 16, 18, 45 and 66

were detected in 65% of the HPV infected tumors. However, no correlation existed between other clinico-pathological variables such as age, gender, sites, habits or histological differentiation and HPV detection when analyzed statistically.

A recent study based on HPV IgG in 78 OPC patients has used Enzyme Linked Immunosorbent Assays (ELISAs) to detect anti-HPV16 and 18 IgG antibodies. It showed a significant risk in developing OPC with the presence of HPV16/18 seropositivity. Total of 46% of the cancer patients were positive for the HPV type 16/18 IgG antibodies. No statistical significant differences were found between age, gender, poor dentition, betel use, smoking, alcohol, and site specificity of the cancer with HPV seropositive and negative OPC patients of this study (Gunasekera *et al*, 2015).

Genotype distribution in HPV associated oral and oropharyngeal carcinomas

Cervical cancers are caused by HR-HPV types 16, 18, 58, 33, 45, 31, 52, 35, 59, 39, 51 and 56 in order of the prevalence. But the genotype distribution of HPV somewhat differs in OPC when compared to carcinoma cervix. A systematic study found the HPV 16 in 95.7% of HPV related OC, but only 73.9% of HPV positive non oropharyngeal HNSCC, but only 61% of cervical cancer shows type 16. Although a significant amount of other high risk types are found in carcinoma cervix, only a small fraction of OPC is caused by other types like 18, 31, 33, 35, 52 and 58 (Gillison *et al*, 2000).

In India HPV 16 is more common than type 18 in HPV associated HNSCC (Nagpal *et al*, 2002).

In a Sri Lankan study done on genotype distribution in OC biopsies, found out HPV type 16 as the predominant type (60% out of total) and type 18 (10%), 45(10%) and 66(3%) as other high risk types(Jayasooriya et al, 2003). In contrast to this, serological study done in oral and pharyngeal carcinoma patients, found out the HPV type 18 as the predominant (32% of total patients) with only 23% of type 16 IgG positivity (Gunasekera *et al*, 2015).

According to Kreimer *et al* (2010), HPV prevalence in normal healthy adults is 4.1% but according to summary report of IARC 2017 it is a range from 0-21% in various studies. In that report, data for Asian countries are not available.

When considering the genotype distribution, oral HPV16, which is the commonest oncogenic type, was found in 1.3% among healthy adults (Kreimer *et al*, 2010).

In the literature, there were no studies in Sri Lanka on oral HPV prevalence and genotype distribution in healthy adults.

But there is evidence in Sri Lanka which shows the community prevalence rate of HPV in cervical smears of normal females as 3.3% with a rate of HPV genotype 16 and 18 as 1.2% (Gamage *et al*, 2009).

2.6 Pathogenesis

HPV accounts for 4.8% - 5.2% of the total global cancer rates, and it is the highest among all oncogenic viruses. This was first suggested in 1983 but first recognized in 1995 and thereafter this virus has been increasingly identified as a major cause for HNSCC, particularly cancers that arise from oropharynx, mostly the base of tongue and palatine tonsils, according to IARC (2007).

Currently there are well established molecular evidence for oncogenic activity of this virus in tumors of the oropharynx with specificity of HPV to the nuclei of tumour cells, integration of viral DNA to the host cell and high HPV copy numbers.

It's clear that HPV infection which leads to cancer requires alterations of host cellular immune reactions. Before viral replication, host immune activation through tumour necrosis factor α (TNFα) and interleukin 1 (IL-1) production may affect the fate of infected cells. And a robust response lead to antiviral activity can down regulate HPV 16 E6/E7 mRNA transcription. At the same time, some studies have suggested the HPV has resistance to TNFα early in the life cycle. From studies involving females with cervical HPV infection, it was shown that most of infected females infected mount an effective immune reaction and ultimately clear the HPV infection. But in about 10%, develop persistent infection, and only a minority (5–10 per 100000) develops malignancy from pre-malignant lesions (Chai *et al*, 2015).

Similar comparisons are still not available in literature for HNSCC. But, evasion from the innate immune system is a hallmark for HPV infection in premalignant and malignant disease and occurs due to viral stimulation and/or repression of the signaling pathways in the host. Another explanation is an evolutionary niche created by the HPV infected keratinocytes, which have a natural short lifespan and are sheltered from large populations of antigen presenting cells. Because of the short keratinocyte lifespan, cell lysis is not necessary for viral escape; but virions are released as the cells propagate through the mucosal layers.

According to the molecular models developed from studies of cervical cancer, there's a suggestion that, oncogenic potential of the virus increases when HPV integrates with the host genome. As a result of HPV integration, oncogenes of the virus will be over expressed which is crucial for oncogenesis(Ragin *et al*, 2006).

E5, E6 and E7 are oncoproteins of HPV but E6 and E7 are the main responsible genes for HPV associated oncogenesis. E6 and E7 genes are able to change the cell cycle so they will keep the differentiating host keratinocyte in a favorable condition for amplification of viral genome and subsequent late gene expression. E6 and E7 have the capacity to bind and inactivate the tumor suppressor proteins p53 and pRb (Retinoblastoma proteins) respectively. These features are associated with the malignant potential of HPV. The E6 protein has zinc binding motif and can make a complex with the p53 protein of the host cell, causing p53 degradation. P 53 is a transcription factor leading to cell cycle arrest/apoptosis in the case of cellular stress/DNA damage(Mistro *et al*, 2013). And also, expression of HR HPV E6 also inactivates telomerase activity, which is an enzyme that maintains telomeric DNA stability(Mannarini *et al*, 2009).

The E7 protein forms complex with retinoblastoma proteins, which is a negative regulator of cell growth which results in the release of the E2F- transcription factors of the cell. The free E2F leads to expression of several host genes involved in the cell cycle progression, and the inactivated p53 and pRb proteins by E6 and E7 permit the cells to bypass normal check points. Collective effects of loss of both p53 and pRb function cause the malignant transformation of epithelial cells(Hill, 2007). Also E7 protein leads

to elevated induction of p16, which is used as a biomarker for HPV associated cancers. The p16 is a tumor suppressor in cells, but is required for the survival of HPV associated cervical cancer cells as well. Oncogenic effect of p16 depends on the inhibition of CDK4/CDK6 in malignant cells where Rb is inactivated, and the presence of CDK4/6 substrates that may cause cell death when phosphorylated in cells with inactivated Rb(McLaughlin *et al*, 2013). The LR-HPV E7 protein binds to pRb with lower affinity than that of HR HPV and do not immortalize the cells.

The E5 is transcribed from the episomal HPV DNA, and the gene is deleted when virus is integrated. Therefore, E5 protein might own its carcinogenic effects during the early phases of the infection. And this viral oncoprotein is not needed for the maintenance of the oncogenic phenotype and according to available data there are no stimulatory effect on cell growth(Ragin *et al*, 2006).

Oncogenic mechanisms in HPV positive HNSCC may be equal to what is described for cervical carcinomas, but since the oral cavity/oropharynx are exposed to increase amount of chemical carcinogens compared to the genital tract, there may be differences in the mechanisms involved in cervical and oropharyngeal oncogenesis(Mistro *et al*, 2013).

2.7 Clinical spectrum

Majority, that is 70–90% of HPV infection is asymptomatic and cleared spontaneously within one or two years. Sometimes HPV can cause persistent infection (detection of specific type of DNA for a period of six months) and rarely can lead to precancerous and cancerous lesions. It will take about twenty years to develop invasive carcinoma since the acquisition of HPV infection (Bonnez, 2017).

Non malignant lesions

Infection with low risk types (90% due to types 6 and 11) causes anogenital warts (condylomata acuminata or venereal warts) which can be difficult to treat and rarely can lead to malignancy. Cutaneous HPV infections are commonly seen in the normal population and they include common warts (commonest type and common in school children), plantar warts (frequent in adolescents and young adults) and juvenile or flat

warts (least common and occur predominantly in children). HPV types 1, 2, 3, 4, 10 are the main etiology for above mentioned cutaneous lesions (Bonnez, 2017).

HPV 6 and HPV 11 can cause a type of lesions of respiratory tract known as recurrent respiratory papillomatosis (RRP). RRP mainly involves larynx and can lead to airway obstruction, which may need surgical intervention. This is mainly acquired by vertical transmission, especially during birth, if mother is having genital HPV infection.

Malignant lesions

HPV infection with HR HPV types are implicated in multiple number of carcinomas of the anogenital region (cervix, anus, vulva, vagina, and penis), gastrointestinal system (esophagus) and head and neck region(Zaravinos, 2014).

Cervical carcinoma

Persistent infections of HPV can progress to premalignant intra-epithelial lesions and cancer. Histopathologically dysplasia of these cervical intra epithelial lesions (CIN) can be categorized as CIN 1- low grade, CIN 2- moderate-marked grade and CIN 3- severe grade dysplasia up to carcinoma in situ. Much of these lesions reduce spontaneously, but some lesions of the cervix can slowly become cancerous over a number of years.

HNSCC

HNSCC are heterogeneous group with tumors in the oral cavity area, oropharynx area, hypopharynx and larynx. Almost 90% of above carcinomas are squamous cell carcinomas. It has poor prognosis, and five year survival is < 50%. Commonest HR genotype which is involved in oncogenesis is type 16 irrespective of the anatomical site of the malignancy.

When considering the anatomy of oral cavity it includes the lip, mucosa of buccal region, hard palate, the anterior 2/3 of tongue, sublingual region, retromolar trigone, alveolar ridges and floor of the mouth.

The oropharynx includes posterior 1/3 (base) of tongue, palatine tonsils, soft palate and posterior/lateral pharyngeal walls(Wangmo *et al*, 2010).

HPV associated HNSCC cancers mainly develop in oropharynx region and affect more frequently the tonsils of palate and posterior 1/3 of the tongue than other oropharyngeal sub sites. There are morphologic characteristics associated with HPV induced carcinogenesis. Clinically, HPV causing OPSCC have characteristic presentation with an earlier T (tumour size) category with a trend for more advanced N (lymph nodes) category compared to HPV unrelated OPSCC. And also patients with HPV associated SCC present with neck metastasis from an occult primary carcinoma. HPV induced lymph nodes metastases are mainly cystic on imaging and regularly undergo sudden changes in volume (Jemal *et al*, 2011; Mistro *et al*, 2013).

HPV induced HNSCC present in relatively young age with mean age at initial diagnosis as 61.1 years, in contrast to 64.5 yrs for HPV unrelated cancers(Hill, 2007).

2.8 Associated factors for HPV infection

These HPV positive tumors have a different profile of risk factors than HPV negative tumors (Fakhry *et al*, 2006). HPV related SCCs are associated with sexual behaviors, oral HPV infection, male gender, higher socio-economic level and immunodeficiency.

HPV is a sexually transmitted infection (STI). Many studies have shown the association between various sexual behaviors, early sexual exposure, number of sexual partners, and HPV positive oral cancers. Risk factors are as the same for cervical cancers, such as number of sexual partners, young age at 1st sexual exposure, oral sex and genital warts (Syrjanen *et al*, 2010).

Although studies on sexual behavior changes are limited, few studies in both Europe and the United States showed that the age of sexual exposure has reduced and number of lifetime sexual partners has increased over the past few decades. The high trend in sexual risk taking (high number of sexual partners and/or more oral sex) in 1960's and 1970's might cause increased HPV exposure (Aral *et al*, 2005; D'Souza *et al*, 2011).

Incidence of both HPV positive and negative HNSCC is more than two times higher in men than women (Chaturvedi *et al*, 2008). High rates of alcohol abuse and smoking in men might be the cause for the increased rates in HPV unassociated HNSCC among men.

But, it's less clear the reason for higher incidence in HPV associated HNSCC in men than women. One hypothesis is a higher HPV burden in the vagina/cervix than the penis, and that person may be more liable to get an oral HPV when doing oral sex with a female than on a male. It is not clear whether this is the only reason, although it is supported from a recent study suggesting the HPV transmission during vaginal sex is commoner from an infected cervix to the penis than the penis to the cervix (Hernandez *et al*, 2008).

Oral HPV infection is also associated with OC and OPC. Oral HPV infection is uncommon (≤1% prevalence) in children (Smith *et al*, 2007), but one study showed higher rates (15%) in infants (Rintala *et al*, 2005), which is measured using HPV DNA in exfoliated oral cells from an oral rinse/swab. Acquisition of HPV is increased around sexual debut with oral HPV infection in 1.5% of 12–15 years old and 3.3% of 16–20 of age group (D'Souza *et al*, 2009; Smith *et al*, 2007). Oral HPV prevalence is higher among adults and it's about 4.5% in healthy adults (Kreimer *et al*, 2010). Oral HPV infection appears to increase with aging, which is unusual for a STI. Possible causes may be, reduced oral HPV clearance with older age or reactivation of latent infections due to age related changes in the immune system (D'Souza *et al*, 2011).

Higher prevalence of oral HPV has been reported in females with cervical HPV infection (Fakhry *et al*, 2006). Current smoking and HIV infection are both associated with significantly high oral HPV prevalence, suggesting that tobacco and HIV related immunosupression may have impact oral HPV natural history making infections more likely to be persistent (Fakhry *et al*, 2006; Kreimer *et al*, 2005).

2.9 Diagnosis

HPV infection can be diagnosed by several methods with different profile of advantages and disadvantages. They include laboratory techniques based on cell morphology, detection of viral proteins and anti-HPV antibodies and detection of HPV genome(Villa, 2006).

Pap smear (Papanicolaou smear), colposcopy and visual inspection can be used to identify the morphology of cells and lesions. This is useful especially in cervical cancer screening programmes.

Immunocito/histochemistry, electron microscopy and Western blot are used for detection of viral proteins. But these methods are technically cumbersome and time consuming.

Above mentioned laboratory techniques based on cell morphology and detection of viral proteins have common limitations like low sensitivity highly rely on sampling and preservation of tissue and difficulty in genotyping.

Up to date, there is no agreed serological test method to detect HPV antibodies as a result of present/past HPV infection. But almost all studies in literature had used enzyme linked immunoassays. Serological methods are available to detect HPV antibodies against L1 VLP (virus like particles) and E6 and E7 antibodies(Burd, 2016). On the other hand, about half the people exposed to HPV never develop measurable levels of antibodies(Villa, 2009) and sometimes it will take several months to develop the detectable level of antibodies. Therefore serological diagnostics methods have reduced sensitivity in comparison to molecular methods.

HPV is difficult to propagate in tissue culture and its accurate identification mainly based on molecular techniques. Therefore molecular assays are considered the gold standard for HPV testing.

Molecular methods have the capacity to identify the many unique genotypes of HR-HPV, resulting in better management of patients. The major molecular techniques for HPV detection are direct detection of genome, probe amplification, signal amplification, and target amplification.

Direct detection of HPV genome and its transcripts is a traditional non amplified technique and it includes southern blot method, *in situ* Hybridization(ISH) and dot blot techniques. Southern blot technique is costly, need large level of purified DNA and ultimately practically difficult to apply in routine practice due to high turnaround time. ISH is not as sensitive as the target and signal amplification methods (Dutra *et al,* 2012).

But ISH tests for HR- HPV can be used to visualize directly the HPV in the tumour cell nuclei in tissue, and helps to demonstrate HPV as the etiology (Burd *et al*, 2016;Villa , 2009).

Ligase chain reaction is a probe amplification method which amplifies the probe.

Hybrid capture assay (HC) is a method that amplifies chemiluminescent or fluorescent signals, rather than the target DNA. It uses a labeled RNA probe to hybridize with the target viral DNA. Multiple conjugated antibodies attached to each captured hybrid and it helps to amplify the signal. In contrast to direct detection methods, these techniques have higher sensitivity and specificity in addition to less dependent on sampling, good for high throughput laboratories and provide viral load data.

Target amplification amplifies DNA fragments and consisted of target sequence. PCR is a very good example for target amplification.

Real-time PCR is a target amplification method with high sensitivity that use fluorescent probes with primers, and able to quantify the virus of the sample accurately. This technique has several advantages 1)can detect the viral load 2) can use to detect multiple genotypes(multiplex genotype PCR) 3) possible to detect DNA at minute concentrations 4) extremely reproducible and rapid – suitable for the clinical setting (Dutra *et al*, 2012).

Diagnosis of HPV in cervical carcinoma

Cytology can be used to detect the changes in the cervical epithelium due to HPV infection. It detects the changes by microscopic examination of exfoliated cells, called Papanicolaou (Pap) test. Pap test is the gold standard for detecting the unusual cervical cells in traditional cervical smears or suspension of cells in liquid cytology medium(Villa, 2009). In low resource settings, visual inspection using acetic acid also helps to screen cervical cancer, according to weekly epidemiological report, WHO (2017). HPV DNA test can be performed on cervical or vaginal swabs and molecular techniques to detect DNA of HR- HPVs are FDA approved for use in combination with cytological methods in screening programmes according to CDC (2015).

Diagnosis of HPV in oral/oropharyngeal carcinomas

Currently there is no recommended technique to screen HPV infection in anus, vulva, vagina, penis, or oropharynx. It's recommended to test HPV in OPSCC as a prognostic indicator. The College for American Pathologists with National Comprehensive Cancer Network suggests the routine laboratory testing for HPV of all OPSCC (Mirghani *et al*, 2014). Reasons for testing are as follows,

1. As a prognostic indicator – HPV positive tumors have better clinical outcomes.

2. For tumor localization – guide to localize the site of primary tumor origin of patients that initially present with metastatic disease (Yasui *et al*, 2014).

3. Distant metastases - rate of distant metastasis in both HPV negative and positive cancers is 5% (Bishop *et al*, 2012) with bulky adenopathy. For HPV negative cancers, metastases occur in lungs and for HPV positive carcinomas, brain, liver, and spine are the possible region for metastases.

HPV detection in OPSCC have a role in management of the patient, and trials to check if OPSCC can be managed less aggressively with the de-escalated treatment than HPV negative tumors are ongoing. Other potential uses are to detect the response for treatment and follow up for recurrence.

Specimen types, collection and transport

Sample type that used for HPV detection can affect the sensitivity and specificity of the final result. Therefore it is important to decide the type of sample carefully in laboratory testing. Commonly used sample type is biopsy sample and ideal is the fresh biopsy. But practically this is not feasible and alcohol fixed or formalin fixed samples are used in practice. As this is an invasive method that needs skills and hospital admission, now there is much attention towards non invasive techniques like cytology. It has the advantages of low cost and also it can be used for cancer screening.

Fine Needle Aspiration Cytology (FNAC) can be used to detect HPV infection in metastatic cervical lymph nodes. Brushing and scraping of cells using cytobrush and

swabs also play an important role in diagnosis but still there are very few studies available in literature on these techniques. But cytology, especially using swabs have proven efficacy in cervical cancer screening and diagnosis of lesions according to WHO (2017).

Dona *et al* (2014) showed in their study that cytobrushing provides a suitable specimen for HPV detection and HPV positivity by cytobrushing was highly associated with the OPSCC diagnosis.

Isaac *et al* (2017) did a study and compare the sensitivity and specificity of oropharyngeal swabs which is a non invasive, painless cost effective method in contrast to biopsies and it showed that swabs can be effectively used for HPV diagnosis and disease surveillance without a biopsy.

Cytology samples collected using oral rinse solutions also has a place in detection of HPV especially in screening outpatient programmes. But oral rinses may not be specific in collection of cells from the tumoral area and not sensitive for diagnosis of malignancy, as it can detect HPV infection which is unrelated to the cancer. In contrast, cytobrushing and swabs can collect site specific representative sample and lead to high specifically. As cytological samples can be collected easily before the biopsy, they are useful to obtain knowledge on HPV status of the lesion rapidly. False negative can occur if the lesion site is difficult to reach or if it is a small tumour(Dona *et al*,2014).

Stability in transportation and storage is highly essential for a good quality DNA. Endogenous endonucleases can breakdown the viral nucleic acid and therefore have to preserve the nucleic acid to avoid false negative results. In molecular techniques it is very important to use control like β-globin to assess the stability and integrity of DNA sample as a part of quality control (Dutra *et al*, 2012).

Laboratory tests

Still there are no standard recommendations for a test or test combinations to identify HPV in OC and OPC. Several techniques with variable strengths and limitations were

used in literature. Commercial tests recommended in cervical cytology can be used after validation by the clinical lab to detect HPV in oropharyngeal specimens(Burd, 2016).

For the diagnosis of HPV associated HNSCC, diagnostic methods detect DNA, RNA or surrogate marker of HPV infection. In situ Hybridization (ISH) for HPV DNA and PCR for HPV DNA and mRNA, and immunohistochemistry (IHC) for a surrogate marker p16 are used alone or in combinations(Tar, 2016).

At present molecular detection can be done by southern transfer hybridization, dot blot hybridization, ISH, HC and PCR in tissue and exfoliated cell samples. But they have different sensitivity and specificity(Zaravinos, 2009). The gold standard for detection of HPV in OPSCC is the quantitative real time PCR to demonstrate HPV DNA (Walline *et al*, 2013).

Presence of HPV DNA in the lesion *per se* doesn't evident the causal relationship, but a viral activity marker is important to diagnose HPV associated carcinoma. Because of the cost and special equipment needed for this method, most laboratories have adopted p16 IHC as the preferred technique of oncogenic HPV detection, and it has become the clinical standard. But, p16 IHC can give rise to false positives as sometimes p16 can be over expressed by HPV independent pathways.

Theoretically analytical 'reference' test for the oncogenic HPV infection is demonstration of transcriptionally active high risk HPV in fresh tissue, usually by reverse transcriptase PCR which amplify high risk HPV E6/E7 mRNA transcripts. Although it is achievable in research laboratories, utility in clinical practice is limited with sub optimal preservation of biopsy samples which contain degraded RNA(Robinson *et al*, 2012, Ang *et al*, 2010). And also reliable IHC probes against E6 and E7 oncoproteins are not yet available (Westra *et al*, 2012).

Various other markers for proliferating cells and biological activity, like proliferating cell nuclear Ag (Ki67), over expression of epidermal growth factor receptor (EGFR), p53 and other markers have been studied. Still none was successful according to current data (Burd, 2016).

Radiologic and histopathologic findings

HPV positive OPSCC are strongly associated with cystic metastatic cervical nodes that can cause misdiagnosis of branchial cleft cyst (Corey *et al*, 2012). Therefore finding of HPV in a cervical metastasis with unknown primary site is a strong indicator of oropharyngeal site for squamous cell cancers. Studies showed distinct radiological profile of cancer and nodal characteristics according to HPV statuses. For an example, HPV positive OPSCC are more likely to have exophytic and well defined borders, and HPV negative OPSCC are likely to show invasion of adjacent structures. In addition, well demarcated cystic metastases are also associated with positivity of HPV in primary tumour (Sood *et al*, 2014).

HPV induced cancers have characteristic histopathological features. They are mainly non keratinized, poorly differentiated SCC or basaloid cancers with lobular growth of cells, hyperchromatic nuclei, scanty cytoplasm and marked mitotic activity.

2.10 Management

Cervical cancer

There is no available specific viral treatment for HPV infection. But screening and treatment for pre-malignant disease is highly helpful to prevent the progression towards cervical cancer. Pre-malignant lesions can be managed using ablative methods to destroy of diseased tissue by burning/freezing with cryotherapy and surgical resection of diseased cells by loop electrosurgical excision procedure (LEEP) or cone biopsy (WHO,2017).

Oral and oropharyngeal carcinomas

Similar to other cancers, treatment options for this cancer group are single modality treatment or multimodality treatment with radiotherapy, chemotherapy surgery as well as targeted therapy in various combinations (Fakhry *et al*, 2006).

Aggressive multimodality therapy can cause high rates of acute as well as long lasting toxicity, which is not suitable for HPV positive patients who are young with high survival rates. At the same time HPV positive cancers show good response to treatment when compared with HPV negative tumors. With this background, more efforts are directed to de-escalate the treatment in HPV associated SCCs to reduce the toxicity and to improve the overall quality of life, while maintaining effectivity (Brizel *et al,* 2012). Several clinical trials are going on about de intensification of current treatment modalities (Mistro *et al*, 2013).

Recently studies mainly focus on reduction of treatment related toxicity and to improve quality of life while maintaining the efficacy of the treatment. They include immunotherapeutic strategies, dose reduction of radiotherapy, and cetuximab instead of cisplatin for chemoradiation and transoral robotic surgery (TORS).

Immunotherapeutic strategies

Commonly local HPV infection can be cleared through immune response of specific T cell mediated immunity. HPV positive OPSCC expresses viral oncoproteins that have immunogenic potential. Most important ones are oncoproteins E6 and E7 and in some studies they have found E7 specific T cells in the circulation.

There are ongoing studies about therapies of HPV positive OPSSC with immunomodulators (toll like receptors), cytokines (IL-12, IL-2, GM-CSF and IFN-α), T cell therapy, and targeted immunotherapy by live viral vector.

Recent studies about immune escape showed an immune resistance mechanism, mediated through programmed death 1 protein (PD-1) with ligand of PD-1 receptor (PD-L1). Usage of PD-1 and PD-L1 antibody in advanced carcinoma patients showed safe and effective results in ongoing studies (Kofler *et al*, 2014).

Dose reduction of radiotherapy

The basis for good outcome seen in HPV associated OPSCC patients is not known. As these patients are young and healthy with better prognosis comparing with HPV negative OPSCC, reducing the long term toxic complications of radiotherapy like swallowing

difficulties, dental dysfunction, speech problems and salivary gland dysfunction is much more important. Dose reduction of radiotherapy also has benefits like reducing the cost of treatment and reduction in time of starting work and unemployment in this young patient group.

Monoclonal antibody therapy

Cisplatin is gold standard agent for therapy for HNSCC but platinum based agents have systemic complications as gastrointestinal, renal, neurologic, otologic and hematologic toxicities. For HNSCC, the approved monoclonal antibody treatment is cetuximab. Replacement of cisplatin with less toxic cetuximab is a possible way to reduce toxicity (Tar, 2016).

Transoral robotic surgery (TORS)

TORS is a new, less invasive, safe procedure leading to reduction in morbidity of HPV positive OPSCC comparing with traditional open surgery methods with lip split and mandible split which have functional impairments. The usage of TORS started in animal models in 2003 and use in humans since 2005. Since then there are many studies published on usage of TORS with good outcomes in OPSCC. The FDA has approved TORS for T1-T2 category of OPSCC. As most patients with HPV positive OPSCC have early T grades, they are considered as good candidates for TORS. Also this is an alternative for chemoradiation for these patients(Tar, 2016).

2.11 Prognosis

A study describing the survival compared to TNM grading in OPC patients found that survival based on TNM status did not relate with survival in HPV positive disease, but it correlated with survival of people with HPV negative disease (Huang *et al*, 2015).

A risk stratification model was produced using HPV status, history of smoking, cancer stage, and nodal involvement. A classification comprised of low, intermediate, or high risk disease has been produced, predicting the 3 year survivals of 93%, 70.8%, and 46.2%, respectively (Ang *et al*, 2010). Ongoing clinical trials show encouraging results on effect of HPV vaccination on prevention and treatment of these tumors (Devaraj *et al*, 2003). In clinical trials, vaccines composed of HPV virus-like particles (VLPs) showed

great effect as prophylactic HPV vaccines. The vaccine has the potential to have a greater impact on these tumour incidences than for cervical cancer incidence, providing the current vaccines are mainly targeted HPV16.

HPV induced SCC had a 54% better survival rates in comparison to HPV negative cancer patients(Mistro *et al*, 2013). According to Ang *et al* (2010), three year rate of disease relapse and metastasis, with the cumulative incidence of the 2nd primary malignancy, were significantly reduced in patients with HPV positive tumors. Furthermore, in contrast to HPV negative patients, HPV positive HNSCCs, OPSCCs and SCCs of tonsils showed a 54%, 53% and 50% reduced levels of mortality, respectively (O'Rorke *et al*, 2012).

The reason for the better prognosis in HPV associated tumours than HPV unrelated tumours remain elusive. Particularly, the good overall survival owned by HPV associated patients may be due to the younger age at initial diagnosis, good performance level, less smoking, less alcohol, distinct tumour biology, less risk of 2nd primary tumors or an aggressive treatment. The favorable result of HPV positive SCC may be due to better sensitivity to treatment of the wild type P53, which showed an apoptotic response of malignant cells to radiotherapy and chemo radiotherapy (Guihard *et al*, 2012).

2.12 Prevention

Cervical carcinoma

Prevention is not a single entity but it is consists of multiple strategies. It includes cancer screening, treatment of premalignant lesions, education about risk behaviours, and more importantly vaccination.

There are three prophylactic vaccines available against HPV. Quadrivalent vaccine (effective against HPV 6, 11, 16, 18) was first licensed in 2006, the bivalent vaccine (for HPV 16, 18) in 2007 and the nonavalent vaccine (for6, 11, 16, 18, 31, 33, 45, 52 and 58) in 2014. All three vaccines consist of purified L1 structural protein prepared by recombinant DNA technology and it is assembled to form HPV type specific empty shell called virus like particles (VLPs). This vaccine is administered through intramuscular

route and indicated before onset of sexual activity. According to current evidence all three vaccines have relatively similar efficacy in prevention of cervical carcinoma (Naud, 2014). A 2 dose regime with a 6 month apart is recommended who receives the 1st dose before the age of 15 years. A 3 dose regime (0, 1–2, 6 months) is indicated for all vaccinations started on or after 15 years of age, including those who are less than 15 years are known to be immunocompromised states.

Bivalent HPV vaccine is used in females and males from the age of 9 years for the prevention of precancerous anogenital disease of cervix, vulval region, vagina, anus, and cancers of cervix and anus related to particular HPV types.

Quadrivalent vaccine is used for women and men from the age of 9 years for prevention of precancerous lesions and cancers of the cervix, vagina, vulval region, and anus caused by HR-HPV types and anogenital warts related to specific genotypes.

Nonavalent vaccine is indicated for males and females since the age of 9 years to prevent the premalignant lesions and malignancies in cervix, vagina, vulva, and anus due to HR-HPV types, and anogenital warts caused by particular HPV types according to HPV vaccines: WHO position paper (2017).

HPV associated HNSCC

Prevention by prophylactic vaccination

These HPV vaccines are indicated for the prevention of cervical cancer with prevention rates about 70%, they may also have potential prophylactic effect on preventing the 95% of HPV positive OPSCC. Although date is available on efficacy against cervical carcinoma, there are no specific data about preventing HPV positive OPSCC. It will take decades for this vaccination programmes to affect the cancer rates of HPV associated HNSCC(Tar, 2016).

Herrero et al showed efficiency of the vaccine as 93% against oral HPV type 16 and 18 infections after 4 years of vaccination in a clinical trial involving Costa Rican females who received either the HPV vaccine (bivalent) or the hepatitis A vaccine as the non-cancer in a random manner. But prevention of HPV associated head and neck cancers is

still not an established indication for HPV vaccine, and more studies are progressing (Chi *et al*, 2015).

Prevention by education and social awareness

As HPV is a sexually transmitted disease, protection methods include condoms and barrier protections during the oral sexual intercourse. Social awareness and education about causal relationship of HPV with OPSCC and possible protection methods must be targeted(Tar, 2016).

According to the available literature, HPV infection associated HNSCC is a global burden. This study will be helpful to find the prevalence, genotype distribution and associated factors of HPV infection in OC and OPC patients of Sri Lanka.

CHAPTER 3

MATERIALS AND METHODS

3.1 Study design

This is a comparative cross sectional study and designed to detect and genotype HPV infection among oral and oropharyngeal carcinoma patients attending Cancer Institute, Maharagama and to describe the associated factors with HPV infection.

3.2 Study setting

Cancer patients were selected from oncology wards (wards 1, 2, 4, 7, 9, 10, 21) and relevant clinics of Cancer Institute, Maharagama and non-cancer group was selected from OPD, National Hospital Sri Lanka (NHSL). Sample processing and molecular diagnosis was done at Medical Research Institute, Colombo 08.

Patients from Cancer Institute Maharagama were selected for the study as it is the main cancer hospital in Sri Lanka for cancer patients and patients from all over the country seek medical service from the Cancer Institute Maharagama.

OPD-NHSL was selected to recruit non-cancer group as large number of patients come to OPD of National Hospital for treatment which facilitated selection of age and sex matched participants.

3.3 Study period

Duration of four months from 15th of December 2015 to 15th of April 2016

3.4 Study population and sample size calculation

Cancer patients group

Patients from oncology wards and clinics (ward 1, 2, 4, 7, 9, 10, 21 and relevant clinics) with histologically confirmed oral and oropharyngeal carcinomas, who didn't undergo any treatment for the current episode were selected for the study during the study period (4 months).

Calculated sample size was 420 according to the probability sampling method. But the estimated number of new patients with oral and oropharyngeal carcinoma patients admitting the Cancer Institute Maharagama was around 150-200 for a period of 4 months according to the available most recent statistical data of the Cancer Institute. Therefore it was practically difficult to take the sample size as calculated from probability method.

According to the convenient sampling method, all patients according to inclusion and exclusion criteria during the study period were included in the study. All the patients meeting above criteria were recruited during study period. Estimated sample size was 150-200 and was able to take 127 participants during the study period.

Patients were selected for the study through their bystanders and Information sheet (Annexure 1) was given and explained the study purpose. Informed written consent (Annexure 2) was taken from the participants, if they wish to participate in the study.

Inclusion criteria

Histologically confirmed oral (OC) and oropharyngeal (OPC) carcinoma patients who haven't undergone any treatment for the current episode from oncology wards and clinics of Cancer Institute, Maharagama (ward 1, 2, 4, 7, 9, 10, 21 and relevant clinics) were recruited during the study period, after considering the exclusion criteria.

Exclusion criteria

Patients who refused to participate in the study, patients who were unable to talk clearly in the interview due to the extensive oral lesions were excluded from the study.

Non-cancer group

Patients from OPD, NHSL who were age and sex matched with cancer patients group were selected as the non-cancer group. Similar number (127) of age and sex matched patients with the cancer patient group were included in non-cancer group.

Similar number of age and sex matched patients with the cancer patients group from OPD-NHSL were recruited according to the inclusion and exclusion criteria.

Information sheet (Annexure 1) was given to patients and explained the study purpose. Informed written consent (Annexure 2) was taken from the participants, if they wish to participate in the study.

Inclusion criteria

Patients who were age and sex matched with the cancer patients group from OPD, NHSL during the study period were selected after considering the exclusion criteria.

Exclusion criteria

People who were refused to participate the study, patients with known risk factors for oral and oropharyngeal cancers such as betel chewing and smoking and people with history of oral and oropharyngeal carcinomas were excluded from the study.

3.5 Study instruments

Cancer patients group

Questions were asked according to the questionnaire (Annexure 3) and other details regarding histological diagnosis, tumor site, tumor staging were taken from bed side notes to the Data Extraction Sheet (Annexure 4) by the Principal Investigator.

Non-cancer group

Same questionnaire (Annexure 3) was used to gather data from OPD patients by the principal investigator.

3.6 Data collection

Cancer patients group

Newly diagnosed oral and oropharyngeal carcinoma patients were identified through caregiver or owner of the patient and using clinical records. Information sheet was given to the patient and explained purpose of the study as well as any questions of patient regarding the study. Informed written consent of the care giver and the patient were taken first in order to select the patient for the study.

Bed side note was used if patient was an inward patient and Clinic Record was used if the participant was a clinic patient to obtain details regarding histological confirmation of the cancer, site of the lesion, staging of the cancer and about the treatment of the disease.

Interviewer based questionnaire was used to gain information on socio-demographic and behavioral data including age, residential area, occupation, marital status, age of 1st sexual exposure, number of sexual partners and habits such as betel chewing, smoking and alcohol consumption.

Data including past history of any other cancer, family history of cancer, time of cancer diagnosis were taken from patient as well as using their above mentioned clinical records.

Non-cancer group

Age and sex matched similar number of people with the group of carcinoma patients were selected from OPD, NHSL, according to inclusion and exclusion criteria.

Information sheet was given to the participant and explained the aim of the study and purpose of the using a non-cancer group. Informed written consent was taken from the participant.

Data regarding age, residential area, occupation, and marital status, age of first sexual exposure, number of partners, family history of cancers, and history of HPV vaccination were gathered using the interviewer based questionnaire.

3.7 Sample collection, processing and storage

Cancer patients group

Samples were taken from the lesion by using oral swabs. Oral swab was a commercial swab with plastic handle and polystyrene tip (flexible long tip which facilitate the sampling) which doesn't inhibit PCR.

The Lesion was thoroughly swabbed and kept immediately in a commercial transport medium with a preservative containing container and labeled with a serial number as documented in the questionnaire.

Samples were transported within 3-4 hours to the Medical Research Institute, Borella. Cold chain (2-8^{0}C) was maintained during sample transportation to the Medical Research Institute, Borella. Samples collection, storage and transport procedures were done according to Universal Precautions.

Non-cancer group

Sixty-three oral and sixty four oropharyngeal swabs were taken from the participant (similar number to the OC and OPC samples from the cancer group) using the commercial swab which was similar to the swab used to collect samples from cancer patients.

As there are no indications for an exact area to be sampled; oral samples were collected swabbing the hard palate, gums, anterior two-thirds of the tongue, and the floor of the mouth and oropharyngeal samples were taken from soft palate, tonsils and base of the tongue (Giuliani *et al,* 2014).

Oral mucosa was thoroughly swabbed and kept immediately inside the commercial transport medium which was similar to the medium used in cancer patients group.

Samples were transported within 3-4 hours to the Medical Research Institute, Borella. Cold chain (2-8^{0}C) was maintained during sample transportation to the Medical Research Institute, Borella. Samples collection, storage and transport procedures were done according to Universal Precautions.

Swab was swirled in the transport medium, squeezed off the fluid inside the tube and swab was discarded. The medium is then stored in two aliquots at -70^{0}C until the extraction.

3.8 DNA extraction(Annexure 5)

DNA extraction was done using a commercially validated HPV DNA extraction kit (RealLine DNA-Express kit - BIORON Diagnostics GmbH), which is compatible with real time PCR assay, used.

Sample, positive control and negative control (100µl each) were added to a tube containing lysis reagent separately. Lysis reagent contains the internal non-cancer DNA which helps to identify PCR inhibition, integrity of the reagents and extraction steps. Incubation of the samples at 98°C in lysis reagent results in cell lysis and release of DNA into solution. Then the sample is ready for the PCR test reaction.

HPV genotyping real time PCR (Annexure 6)

RealLine HPV High Risk Genotype Fla-Format - BIORON Diagnostics GmbH, a commercially validated qualitative kit for the differential determination of DNA of 12 HR- HPV types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59) by Real Time PCR method. Verification of the kit using samples with known HPV 16 and 18 was also carried out prior to testing of clinical samples in the study (Annexure 11).

The sensitivity and specificity of the kit was 100%.The kit was stored at 4-8°C on arrival as per instructions in the manual and aliquots were made using the positive non-cancers, negative non-cancers and recovery solution to prevent freeze-thaw cycles of more than two and to reduce the risk of contamination.

To analyze each sample for the content of HPV DNA, four tubes were used along with each Master Mix (MM1, 2, 3, 4) for detection of DNA (see Table 1).

Table 1Master mixture types

Test-tube	HPV types
MM1	16, 18, 39
MM2	33, 45, 56
MM3	31, 35, 58
MM4	52, 51, 59

Master mixture set up, reaction set up, programming the Real-Time PCR instrument, assessing the validity of the test run and interpretation of results were carried out according to manufacturer's instruction. Each PCR run consisted of four positive controls (MM1-4), four negative controls (MM1-4), no template non-cancer, together with the sample (four tubes per one sample with MM1-4).

The PCR was carried out using Applied Bio systems 7500 Real-Time PCR system and the used RealLine HPV High Risk Genotype kit was validated to be used with the instrument (Annexure 10).

Amplification of target HPV DNA was detected by using target specific probes labeled with fluorescent reporter dyes. Genotypes 16, 33, 31 and 52 were detected by FAM. Genotypes 39, 56, 58 and 59 were detected by JOE while type 18, 45, 35 and 51 were detected in ROX. Internal control (MM 1, 2, 3) amplification was detected by Cy5.In order to validate the quality of sampling and improve the reliability of results of High Risk HPV DNA (MM 4) detection, the content of human DNA (β-actin) is additionally estimated in the analyzed samples, in Cy5.

The results of the genotyping PCR were informed to the consultant in-charge and the relevant patients in the form of a report.

3.9 Statistical analysis

Data were analyzed using Statistical Package for Social Sciences version (SPSS) 21.

Descriptive statistical methods were used to analyze demographic data and percentages of HPV infection in both groups. Univariate analyses and Multivariate analysis were used to compare selected variables between cancer patients and non-cancer group. Chi Square and Fisher's Exact Tests with p value of 0.05 were used to analyse the association between selected variables and HPV infection among cancer patients.

3.10 Ethical consideration and confidentiality

Ethical approval was obtained from the scientific and ethical review committee at MRI (project no–27/2015) (Annexure 7) Director and relevant consultants of Cancer Institute,

Maharagama (Annexure 8) and Ethical review committee at NHSL (Annexure 9). Swab samples and data were collected after obtaining written informed consent from the participants and patient confidentiality was strictly maintained by not including their names in the data collection form.

CHAPTER 4

RESULTS

4.1 Basic socio-demographic characteristics of the study subjects

Males were predominant (95.3%) among both cancer and non-cancer groups. Highest proportion (44.1%) was of age 51-60 years in both cancer and non-cancer groups. Least proportion (3.1%) of both groups was in 31-40 years (Table 2). Education level of grade 6-11 was predominant among cancer and non-cancer cohorts (45.6% and 39.4% respectively). Degree was the least predominant (3.4% and 1.6%) among cancer patients and non-cancer people respectively (Table 3).

Highest proportion of study subjects (87%) was married. 9.8% of study subjects were unmarried. 3.2% of study subjects were separated (Table 4). Labourers were the predominant employment category (28.3%) (Table 5). All the study subjects were not received vaccination against HPV. Highest proportion (70.9%) of study subjects had the first sexual exposure at the age category 20-30 years. 6.6% study subjects had not have a sexual experience.

Table 2 Age distribution

Age	Cancer patients n (%)	Non-cancers n (%)	Total n (%)
< 20yrs	0(0)	0(0)	0 (0)
21-30yrs	0(0)	0(0)	0(0)
31-40yrs	4(3.1)	4(3.1)	8(3.2)
41-50yrs	18(14.2)	18(14.2)	36(14.2)
51-60yrs	56(44.1)	56(44.1)	112(44.1)
61-70yrs	32(25.2)	32(25.2)	64(25.2)
>70yrs	17(13.4)	17(13.4)	34(13.3)
Total	**127(100)**	**127(100)**	**254(100)**

Table 3 Gender distribution

Gender	Cancer patients n (%)	Non-cancers n (%)	Total n (%)
Males	121(95.3)	121(95.3)	242(95.3)
Females	6(4.7)	6(4.7)	12(4.7)
Total	**127(100)**	**127(100)**	**254(100)**

Table 4 Education level

Education	Cancer patients n (%)	Non-cancers n (%)	Total n (%)
Not schooled	14(11)	28(22)	42(16.5)
Grade 1-5	42(33)	36(28.3)	78(30.7)
Grade 6-11	58(45.6)	50(39.4)	108(42.5)
Up to A/Level	9(7)	11(8.7)	20(7.9)
Degree	4(3.4)	2(1.6)	6(2.4)
Total	**127(100)**	**127(100)**	**254 (100)**

Table 5 Marital status

Marital status	Cancer patients n (%)	Non-cancers n (%)	Total n (%)
Unmarried	16(12.6)	9(7.1)	25(9.8)
married	107(84.2)	114(89.8)	221(87.0)
Separated	4(3.2)	4(3.1)	8(3.2)
Total	**127(100)**	**127(100)**	**254(100)**

Table 6 Age at first sexual exposure among study subjects

Age at first sexual exposure	n	%
<20yrs	22	8.7%
20-30yrs	180	70.9%
>30yrs	35	13.8%
Not exposed	17	6.6%
Total	**254**	**100%**

Table 7 Employment category

Employment category	n	%
Businessman	26	10.2%
Carpenter	11	4.3%
Clerk	7	2.8%
Conductor	1	0.4%
Driver	33	13.0%
Electrician	5	2.0%
Farmer	29	11.4%
Fisherman	17	6.7%
GramaNiladari	1	0.4%
House wife	8	3.2%
Labourer	72	28.3%
Mason	19	7.5%
Mechanic	13	5.2%
Police Constable	1	0.4%
Postman	1	0.4%
Railway officer	1	0.4%
Security officer	2	0.8%
Self employed	2	0.8%
Teacher	4	1.6%
Tourist guide	1	0.4%
Total	**249***	**100%**

*Occupation data was missing in 5 study subjects.

4.2 Prevalence of HPV among oral and oropharyngeal carcinomas

Cancer group consisted of 63 oral carcinoma patients (50%) and 64 oropharyngeal carcinoma patients (50%).

Among the oral cancer patients 7 (11%) were HPV infected. Among oropharyngeal cancer patients 12 (19%) were HPV infected. Among the total cancer patients 19 (15 %) were HPV infected and 108 (85 %) were not infected.

Table 8 Prevalence of HPV infection

Cancer type	**HPV infected n (%)**	**HPV not infected n (%)**	**Total n (%)**
Oral	07 (11)	56(89)	63(100)
Oropharyngeal	12(19)	52(81)	64(100)
Total	**19(15)**	**108(85)**	**127(100)**

4.3 HPV genotypes and its distribution among oral and oropharyngeal carcinoma patients

Table 9 HPV genotype distribution among cancer group

Cancer type	HPV types			
	Type 16 n (%)	**Other high risk types n (%)**	**Not infected n (%)**	**Total n (%)**
OC	4(6.2)	3(4.6) **Genotypes** 1.Dual 39,59 2. 59 3. 52	56(89.2)	63(100)
OPC	7(11)	5(8) **Genotypes** 1. 58 2. 52, 58 3. 52 4. 52 5. 52	52(80.7)	64(100)
Total	**11(8.7)**	**8(6.3)**	**108(85.0)**	**127(100)**

Among oral cancers 4(6.2%) were infected with HPV Type 16; 3(4.6%) infected with other HR-HPV types. HPV genotypes 39, 52 and 59 were detected in the infected oral carcinoma patients group. One patient had a dual infection with HPV genotypes 39 and 59. Fifty six (89.2%) of oral cancer patients were not infected with HPV.

Among oropharyngeal cancer patients 7(11%) were infected with HPV Type 16; 5 (8%) were infected with HPV risk types. Genotype 52 was detected in 4 study subjects and type 58 was detected in 2 subjects. One patient had a dual infection with genotypes 52 and 58. Fifty two (80.7%) oropharyngeal patients were not infected with HPV.

HPV Type 16 proportion (8.7%) is higher than other high risk types (6.3%). Altogether type 16 accounts for 58% of total HPV infection. Majority (85%) of patients with oral and oropharyngeal carcinomas were not infected with HPV. HPV Type 18 infection was not detected in this study population.

4.3 HPV detection and genotype distribution among non-cancer group

HR-HPV types were detected in 4 of (3.1%) non-cancer group. Genotype 59 was detected in 1 subject and genotype 52 was detected in other 3 subjects. Types 16 and 18 were not detected.

4.4 Results of univariate analysis – associations of oral and oropharyngeal carcinomas

Table 10 Association between education level and oral and oropharyngeal carcinoma

Education level	Carcinoma (oral/oropharyngeal)				Total	
	Present		Absent			
	n	%	n	%	n	%
Not schooled	14	11	28	22	42	16.5
Schooled	113	89	99	78	212	83.5
Total	127	100	127		254	100

OR 0.438 95% CI 0.218-0.879 p value 0.02

There is a statistically significant association between education level and (oral and oropharyngeal) carcinoma.

Table 11 Association between dental hygiene and oral and oropharyngeal carcinoma

Dental hygiene	Carcinoma (oral/oropharyngeal)				Total	
	Present		Absent			
	n	%	n	%	n	%
Good	24	18.9	68	53.5	92	36.2
Bad	103	81.1	59	46.5	162	63.8
Total	127	100	127	100	254	100

OR 0.202 95% CI 0.115-0.356 p value 0.00

There is a statistically significant association between dental hygiene and carcinoma (oral and oropharyngeal).

Table 12Association between betel chewing and oral and oropharyngeal carcinoma

Betel chewing	Carcinoma (oral/oropharyngeal)				Total	
	Present		Absent			
	n	%	n	%	n	%
Yes	77	60.6	0	0	77	30.3
No	50	39.4	127	100	177	69.7
Total	127	100	127	100	254	100

OR 3.54 95% CI 2.79- 4.48 p value 0.00

There is a statistically significant association between betel chewing and (oral and oropharyngeal) carcinoma.

Table 13Association between smoking and oral and oropharyngeal carcinoma

Smoking	Carcinoma (oral/oropharyngeal)				Total	
	Present		Absent			
	n	%	n	%	n	%
Yes	89	70.1	0	0	89	35
No	38	29.9	127	100	165	65
Total	127	100	127	100	254	100

OR 4.3495% CI 3.29-5.74 p value 0.00

There is a statistically significant association between smoking and (oral and oropharyngeal) carcinoma.

Table 14Association between alcohol and oral and oropharyngeal carcinoma

Alcohol	Carcinoma (oral/oropharyngeal)				Total	
	Present		Absent			
	n	%	n	%	n	%
Yes	89	70.1	107	84.3	196	77.2
No	38	29.9	20	15.7	58	22.8
Total	127	100	127	100	254	100

OR 0.44 95% CI 0.24-0.81 p value 0.01

There is a statistically significant association between alcohol consumption and (oral and oropharyngeal) carcinoma.

Table 15Association between history of warts and oral and oropharyngeal carcinoma

History of warts	Carcinoma (oral/oropharyngeal)				Total	
	Present		Absent			
	n	%	n	%	n	%
Present	3	2.4	4	3.1	7	2.8
Absent	124	97.6	123	96.9	247	97.2
Total	127	100	127	100	254	100

OR 0.74 95% CI 0.16-3.39 p value 1.0

There is no statistically significant association between history warts and (oral and oropharyngeal) carcinoma.

Table 16Association between HPV infection and oral and oropharyngeal carcinoma

HPV infection	Carcinoma (oral/oropharyngeal)				Total	
	Present		Absent			
	n	%	n	%	n	%
Yes	19	15	4	3.1	23	9
No	108	85	123	96.9	231	91
Total	127	100	127	100	254	100

OR 5.0895% CI 1.67-15.47 p value 0.003

There is a statistically significant association between HPV infection and (oral and oropharyngeal) carcinoma.

Table 17Association between number of sex partners and oral and oropharyngeal carcinoma

HPV infection	Carcinoma (oral/oropharyngeal)				Total	
	Present		Absent			
	n	%	n	%	n	%
Yes	118	92.9	123	96.9	241	94.9
No	9	7.1	4	3.1	13	5.1
Total	127	100	127	100	254	100

OR 0.43 95% CI 0.13-1.42 p value 0.25

There is no statistically significant association between number of sexual partners and (oral and oropharyngeal) carcinoma.

Table 18Association between age and oral and oropharyngeal carcinoma

Age*	Carcinoma (oral/oropharyngeal)				Total	
	Present		Absent			
	n	%	n	%	n	%
<51 yrs	22	17.3	22	17.3	44	17.3
≥51 yrs	105	82.7	105	82.7	210	82.7
Total	127	100	127	100	254	100

OR 1 95% CI 0.522-1.915 p value 1

*ROC curve was used to determine the cutoff point

There is no statistically significant association between age and (oral and oropharyngeal) carcinoma.

Table 19Association between gender and oral and oropharyngeal carcinoma

Gender	Carcinoma (oral/oropharyngeal)				Total	
	Present		Absent			
	n	%	n	%	n	%
Female	6	4.7	6	4.7	12	4.7
Male	121	95.3	121	95.3	242	95.3
Total	127	100	127	100	254	100

OR 1 95% CI 0.314-3.188 p value 1

There is no statistically significant association between gender and (oral and oropharyngeal) carcinoma.

Table 20 Association between age at first sex and oral and oropharyngeal carcinoma

Age at first sex	Carcinoma (oral/oropharyngeal)				Total	
	Present		Absent			
	n	%	n	%	n	%
not experienced First sex	9	7.1	8	6.3	17	6.7
Experienced first sex	118	92.9	119	93.7	237	93.3
Total	127	100	127	100	254	100

OR 1.135 95% CI 0.423-3.041 p value 1.08

There is no statistically significant association between age at first sex and (oral and oropharyngeal) carcinoma.

4.5 Multivariate logistic regression

Multivariate logistic regression analysis was performed for controlling the confounding factors.

Table 21 Multivariate regression

Variables	B coefficient	Standard error	OR	95% CI	p value
Education level	-.254	1.026	.775	0.104 - 5.789	0.804
Dental hygiene	.445	.755	1.561	0.355-6.861	0.555
Betel chewing	-51.162	4494.818	1.21	1.45-19.67	0.04
Smoking	-52.263	4476.416	1.56	2.67-16.65	0.03
Alcohol consumption	32.493	2981.088	1293.8	1078.34-1789.61	0.04
HPV infection	-16.261	2060.674	1.27	1.08- 27.87	0.05

Significant level - P value was set at ≤ 0.05

Betel chewing, smoking, alcohol consumption and HPV infection showed statistically significant association with oral/oropharyngeal carcinoma.

Education level, dental hygiene did not show statistically significant association with oral/oropharyngeal carcinoma.

4.6 Association between selected variables and HPV infection status among cancer patients

Sub analysis was performed among cancer cases to examine the association between age, gender, age at first sex, histology, tumour size, lymph nodes, recurrence and type of cancer (oral/oropharyngeal) and HPV infection. Significant level (p value) was set at ≤ 0.05.

Table 22 Association of selected variables and HPV infection status among cancer patients

Variable	Chi Square value	df	p value	Statistical significance (S*/NS*)
Gender	**		0.61	NS
Age	2.746	1	0.09	NS
Age at first sex	1.728	1	0.19	NS
Smoking	131.83	4	0.00	S
Education level	137.82	10	0.00	S
Dental hygiene	133.94	4	0.00	S
Betel chewing	131.233	4	0.00	S
History of warts	104.542	4	0.00	S
No. of sexual partners	1.988	2	0.37	NS
Histology	1.047	4	0.90	NS
Tumour size	37.192	18	0.005	S
Presence of lymph nodes	109.69	10	0.00	S
Recurrence	132.51	4	0.00	S
Oral/oropharyngeal type	88.64	4	0.00	S

**Fisher's Exact Test

S*/NS*- significant/non significant

Smoking, education level, dental hygiene, betel chewing, history of warts, tumour size, presence of lymph nodes, recurrence and oral/oropharyngeal cancer types showed significant association with HPV infection among cancer cases.

CHAPTER 5

DISCUSSION

This comparative cross sectional study was conducted among two cohorts including oral and oropharyngeal cancer patient group and non-cancer group, who don't have oral and oropharyngeal cancer lesions. The cancer patient group was selected from Cancer Institute Maharagama, which is the leading national hospital that treats cancer patients from all over the country.

Swabs of the lesions were collected from patients and genotyping real time PCR (rt-PCR) assay was used to detect HPV DNA. The Gold standard method to detect HPV in cancer patients is detecting HPV DNA in tissue biopsies or exfoliated cells by molecular diagnostic methods like rt-PCR (Isaac, 2017). Swabs were used to collect the exfoliated cells of the lesion, which is a non invasive, feasible method. The assay used for rt-PCR for this study contained the internal control including human beta gene which is a prerequisite for test validation which was designed to detect human cells of the specimen. This increases the sensitivity of the test by reducing the false negatives due to improper collection of sample and also it ensures the integrity of the DNA of virus during the sample storage and transport.

HR-HPV was detected in 15% of the total oral and oropharyngeal cancer patients. 11% of oral cancer patients and 19% of oropharyngeal cancer patients were positive for HR-HPV infection. Worldwide, HPV infection in oral and oropharyngeal carcinoma patients varied according to the geographical area and it ranged between 45-90% mostly due to differences in sample sizes as well as sensitivity and specificity of testing methods used for studies (D'Souza *et al*, 2007; Kreimer *et al*, 2005).

HPV attributed fraction is about 7% to 16% for oral carcinomas and 40% for oropharyngeal carcinomas in developed countries (Chi *et al*, 2015). In Asian countries, rates are much higher, 33% and 46.3% of oral and oropharyngeal carcinomas respectively (Kreimer *et al*, 2005).

HPV is mainly a sexually transmitted infection and HPV associated cancers are increasing globally due to changing pattern of sexual practices. Cultural differences between other countries and Sri Lanka may be a contributing factor for the low rates observed in this study compared to global figures. All most all studies on HPV in oral and oropharyngeal carcinoma patients had used biopsies of the lesions for HPV detection. But in this study swabs were used to collect samples and not much published data available to compare the results. Therefore this study will be a good starting point for future studies to compare the sensitivity of using swabs and biopsies as sampling methods to detect HPV.

In Sri Lanka Gunasekara *et al* (2015) had done a study on HPV IgG in oral and oropharyngeal carcinoma patients and detected 46% of high risk HPV. As it was a serological study which used in house ELISA test, specificity is very much low and there can be false positives in contrast to rt-PCR which has 100% sensitivity and specificity (Castro *et al*, 2006). Another local study done by Jayasooriya *et al* (2003), found out the prevalence of 37.2% of HPV in formalin fixed biopsies taken from oral carcinoma patients which was tested by PCR.

As HPV can also be seen in normal oral mucosa, detection of HPV in a non-cancer group was performed to see the statistical significance of the presence of HPV on oral and oropharyngeal carcinoma patient group. Among the non-cancer group, 3.1% were infected with HPV, which is compatible with global rates of 4.5% in healthy adults (Kreimer *et al*, 2010). There are no published data on prevalence of HPV in oral mucosa of normal population in Sri Lanka before this study.

According to IARC, there are 12 HR-HPV types associated with human cancers (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59) and all those 12 types were included in the genotyping rt-PCR assay that was used in this study. This commercial assay was verified in house and also validated for the used PCR machine to ensure the maximum quality of HPV genotype detection.HPV Type 16 proportion was higher than other high risk types 39, 52, 58, 59 in HPV positive cancer patient group. Altogether type 16 accounts for 58% of total HPV infection with rest of 42% due to other HR-HPV types.The HPV Type 18

infection was not detected in this study population. Globally, HPV type 16 accounts for >90% with regard to genotype distribution(Kingma *et al*, 2010).Other HR-HPV types;18, 33, 35, 45 and 58 were trivial comparatively (Zaravinos, 2014).

The study done by Gunasekara *et al* using an in house ELISA assay (in which validation and verification details are not clearly mentioned in the paper) on seventy eight oral and pharyngeal cancer patients, found HPV type 18 IgG in 32% of patients (Gunasekara *et al*, 2015) in contrast to global/regional seroprevalence data of type 16 as the predominant (Tiggelaar *et al,* 2012).However, IgG seropositivity indicates past/recent exposure to HPV and not indicative of viraemia or viral infection of the lesion.

In the non-cancer group, genotype distribution consisted of HR-HPV types 52 and 59. Interestingly, genotypes 16 and 18 were not found in this group in contrast to global condition with HPV type 16 of 1.3% out of 4.4% of the total HPV infection in healthy people (Kreimer *et al*, 2010).

There was one oral carcinoma patient with dual infection of 39 and 59 genotypes and one oropharyngeal carcinoma patient with types 52 and 58. Dual infections were found in some other studies as well but significance of this is still debatable in relation to oncogenesis(Kingma *et al*, 2010), and further studies should be done on this direction to find out the importance of other high risk types and dual infection in carcinogenesis. This will help to guide and improve vaccine composition for HPV prevention related to oral and oropharyngeal carcinomas in future.

With the above mentioned figures on HR-HPV in oral and oropharyngeal carcinoma patient group and non-cancer group of this study, univariate and multivariate analysis showed statistical significant association of HR-HPV infection with oral and oropharyngeal carcinoma patients, in addition to betel chewing and smoking. This is compatible with global status (IARC, 2016) and in other studies of Asian countries like India and Taiwan (Rao *et al*, 2013). In Sri Lanka few studies were done on this area and according to Siriwardhana *et al* (2006), socioeconomic status and smoking are related with oral carcinomas. Although there is a statistically significant association, data suggested a reverse protective effect than the expected finding of alcohol consumption

leading to oral and oropharyngeal cancers in literature (Rao *et al*, 2013).Difficulty in drinking alcohol with severe mucosal lesions in later stage of cancer may be the contributing factor and may need further studies to clarify this finding.

Knowledge on associated factors for HPV infection in oral and oropharyngeal carcinomas facilitate the identification of oral and oropharyngeal carcinoma patients with HPV infection who should undergo HPV diagnosis during the clinical assessment.

Interviewer based questionnaire and clinical records were used to gather information regarding sociodemographic and behavioral factors. The Chi Square and Fisher's exact tests were used to analyze and to describe the associated factors for HPV infection in oral and oropharyngeal carcinoma patients. Education level, poor dental hygiene, smoking, betel chewing, large tumour size, presence of lymph nodes, primary tumour without recurrence and oropharyngeal carcinoma type showed significant association with HPV infection among OC and OPC patients, and it is compatible with global condition(Chaturvedi *et al*, 2008; Syrjanen *et al*, 2010)

Age, gender, age of first sexual exposure, and number of sexual partners showed no significant association with HPV infection in this study. But, globally HPV infection is associated with younger age, male gender, and number of life time sexual (Chaturvedi *et al*, 2008). This difference may be due to cultural concerns of the society and not openly coming out with correct information on sexual behaviors, especially when using an interviewer based questionnaire.

These study findings will be helpful to see the changes on prevalence of HPV infection in oral and oropharyngeal carcinoma patients as well as in normal population after introduction of HPV vaccination to the national immunization schedule. Significant HR-HPV infection in male patients with oral and oropharyngeal carcinomas highlight the importance of considering the introduction of HPV vaccine to the male population in future, as at present primary target group is only girls, in national immunization schedule. Findings of this study convince the importance of screening of OC and OPC patients for HR-HPV before starting treatment, as HPV positive patients have good response to

treatment (can manage with de-escalated treatment), less recurrence, good prognosis with overall improved survival rates.

Limitations of the study

Small sample size is a limitation of this study due to limited time factor.

Conclusion

HR-HPV infection was detected in 15% of oral and oropharyngeal cancer patients and HR-HPV infection was significantly associated with oral and oropharyngeal carcinoma patients. Oral and oropharyngeal carcinoma patients were infected with HR-HPV types 16, 39, 52, 58 and 59 and the type 16 was the most prevalent genotype. HPV infection was associated with multiple socio demographic, behavioral and tumor factors. Education level, poor dental hygiene, smoking, betel chewing, large tumour size, presence of lymph nodes, primary tumour without recurrence and oropharyngeal cancer type showed significant association with HPV infection among oral and oropharyngeal cancer patients.

Recommendations

Further longitudinal studies should be done to find out the prognosis and treatment response of the HR-HPV positive patients. It is essential to follow the normal people who were positive for HR-HPV in the non-cancer group to see the clearance/persistence of HPV infection and association with oncogenesis.

Future studies with high sample volume are recommended to find out the prevalence and genotype distribution of the HPV infection in oral and oropharyngeal carcinoma patients.

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ANNEXURE 1c

INFORMATION SHEET

Title of the project : Detection and genotyping of Human Papillomavirus in patients

with oral and oropharyngeal carcinoma at the Cancer Institute,Maharagama.

Name of the investigator : Dr.Buddhini Samaraweera MD Trainee, MRI, Colombo 08.

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Name of the supervisor : Dr.J.I.Abeynayake, Consultant virologist, MRI, Colombo 08.

Human Papillomavirus is a virus which can cause cancers in humans such as cancers of mouth and throat. Cancers of mouth and throat caused by Human Papillomavirus have different methods of diagnosis, treatment and prevention. And also people get Human Papillomavirus infection due to various reasons. In world, experiments are going on to find out whether vaccination against Human Papillomavirus can prevent above carcinomas.

So, my project will help to detect Human Papillomavirus in mouth and throat and classify them by taking oral swab samples. And also it will help to find out the causes for getting Human Papillomavirus using information gathered from asking questions.

As similar studies has not been done in Sri Lanka, results of this project will be helpful in improving knowledge on diagnosis, treatment and prevention of cancers of mouth and throat.

The relevant clinicians and participants will be informed about results and also about the good prognosis and survival of Human Papillomavirus positive tumours.

තොරතුරු පත්‍රිකාව

පර්යේෂණ මාතෘකාව - මහරගම පිළිකා රෝහලේ මුඛය හා උගුර ආශ්‍රිත පිළිකා රෝගීන්ගේ ''හියුමන් පැපිලෝමා වයිරසය'' ඇත්දැයි බැලීම හා එය ජානමය වර්ගීකරණයට ලක් කිරීම.

ප්‍රධාන පර්යේෂක :- වෛද්‍ය බුද්ධිණී සමරවීර

වෛද්‍ය පර්යේෂණ ආයතනය, බොරැල්ල.

ජංගම දුරකථනය - 071-4495458

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අධීක්ෂකතුමාගේ නම් - වෛද්‍ය J. I. අබේනායක

වයිරස් පිළිබඳ විශේෂඥ වෛද්‍ය,

වෛද්‍ය පර්යේෂණ ආයතනය,

බොරැල්ල.

''හියුමන් පැපිලෝමා වයිරසය'' නමැති වයිරසයට මිනිසුන්ගේ මුඛය හා උගුර ආශ්‍රිත පිළිකා ඇති කිරීමට හැක. මුඛය හා උගුර ආශිත පිළිකා ඇති කරන එම වයිරසය සඳහා විවිධ රෝග හඳුනා ගැනීමේ ක්‍රම, ප්‍රතිකාර හා වළක්වා ගැනීමේ ක්‍රම ඇත. මෙම වයිරසය විවිධ හේතු නිසා මිනිසුන්ට ආසාදනය විය හැක. ලෝකයේ මේ වන විට එන්නත් මගින් මෙම විශේෂත පිළිකා කාණ්ඩය වළකා ගත හැකි බවට පර්යේෂණ පවත්වමින් තිබේ.

මාගේ පර්යේෂණයේ දී පිළිකා රෝගීන්ගේ මුව සෝදා ලබා ගන්නා දියර සාම්පලය යොදා ගෙන එම වයිරසය සිටී දැයි සොයා ගැනීමට හා එම වයිරසය වර්ගීකරණය කළ හැක. එමෙන්ම එම රෝගීන්ගෙන් අසන ප්‍රශ්න මගින් එම වෛරසය ආසාදනය වීමට හේතු පිළිබඳ තොරතුරු ලබා ගත හැක.

මේ හා සමාන පර්යේෂණ දැනට ශ්‍රී ලංකාවේ සිදු කර නොමැති නිසා මාගේ පර්යේෂණයෙන් ලබා ගන්නා තොරතුරු මෙවැනි මුඛය හා උගුර ආශ්‍රිත පිළිකා රෝග විනිශ්චය ප්‍රතිකාර ක්‍රම හා වළක්වා ගැනීමේ ක්‍රම පිළිබඳ දැනුම වැඩි දියුණු කර ගැනීමට උපකාරී වේ. මෙහි ප්‍රතිඵල අදාල වෛද්‍යවරයාට හා රෝගියාට දන්වන අතර ''හියුමන් පැපිලෝමා වයිරසය'' නිසා ඇති වන පිළිකා රෝගයේ සාමාන්‍ය ලෙස ජීවත් වීමට ඇති කාලය හා පිළිකා රෝගය නිසා ජීවිත හානියක් සිදු නොවී ජීවත් වීමට ඇති කාලය වැඩි බවද දැනුවත් කෙරේ.

No :_____ Date: __/__/____

தகவல் பத்திரம்.

திட்டத்தின் தலைப்பு : முகாமையாளர் புற்று நோய் நிறுவனத்தின் வாயும், வாய் தொண்டை தொற்றான புற்று நோய் நோயாளர்களின் மனித பபிலோமா வைரஸ் ஜீனோடைபிங்கை (HPV) கண்டறிதல்.

ஆராய்ச்சியாளரின் பெயர் : வைத்தியர் புத்தினி சமரவீர
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மருத்துவ ஆய்வுகூட நிலையம்,
கொழும்பு - 08.

மனிதனின் தொண்டை, நாக்கில் புற்றுநோயை ஏற்படுத்தும் வைரஸ் மனித பபிலோமா வைரஸ் ஆகும். ஆனால், தொண்டையில் புற்று நோயை ஏற்படுத்தும் மனித பபிலோமா வைரஸை (HPV) பல முறைகளில் கண்டறிந்து சிகிச்சை அளித்து தடுக்க முடியும். பல காரணங்களினால் இவ்வைரஸ் தொற்று ஏற்படுகின்றது. இந்த உலகில் இவ்வைரஸிற்கான தடுப்பூசி போட்டு புற்று நோயை கட்டுப்படுத்த முடியுமா என பல ஆராய்ச்சிகள் நடந்து கொண்டிருக்கின்றது. அதையால் எனது இத்திட்டத்தின் மூலம் வாய், தொண்டையிலிருந்து எடுக்கப்படும் மாதிரியினால் இவ்வைரஸை (HPV) கண்டறிய உதவும். வைரஸ்கள் கேட்பதன் மூலம் கிடைக்கும் தகவல்களினால் இவ்வைரஸ் ஏற்படும் வழிகளை கண்டறிய முடியும். இவ்வாறான ஆராய்ச்சிகள் இலங்கையில் இல்லை. இத்திட்டமானது வாய், தொண்டையில் ஏற்படும் புற்றுநோயை கண்டறியவும், சிகிச்சை

No : _____ Date: __/__/____

அளிக்கவும், தடுக்கவும் உதவியாக இதனால் கிடைக்கும் அறிவு போதுமானதாகவும் மேம்பாட்டுக்கும் உதவும்.

ANNEXURE 2c

CONSENT FORM

Serial number: [ ]

Title of the project : Detection and genotyping of Human Papillomavirus in patients with oral and oropharyngeal carcinoma at the Cancer Institute,Maharagama.

Name of the investigator : Dr.Buddhini Samaraweera.

Contact details- mobile 071-4495458

E mail buddiyasasi@gmail.com

Name of the supervisor : Dr.J.I.Abeynayake, Consultant virologist, MRI, Colombo 08.

I,..... have been informed about the purpose of the study "Detection and genotyping of Human Papillomavirus in patients with oral and oropharyngeal carcinoma at the Cancer Institute, Maharagama" which will be carried out as a part of postgraduate training of Dr.Buddhini Samaraweera and I am fully informed and understand that no harm or injury will occur to me during the collection of oral swab sample which is used to detect Human Papillomavirus.

I am aware that the information gathered from this study will be used for research purposes only and will not be divulged to any other party and the confidentiality is maintained during the study period and my identity will not be revealed in the publications of the results.

I am fully aware that I have the complete autonomy and liberty of refraining from consenting for this study and even withdrawing from the study after consenting.

I hereby consent for the above mentioned study.

Care giver/ owner of the patient

Participant Investigator Date

අවසර පත්‍රිකාව

රෝගියාගේ අංකය : [ ]

පර්යේෂණ මාතෘකාව - මහරගම පිළිකා රෝහලේ මුඛය හා උගුර ආශ්‍රිත පිළිකා රෝගීන්ගේ ''හියුමන් පැපිලෝමා වයිරසය'' ඇත්දැයි සොයා බැලීම හා එම වයිරසය ජානමය ලෙස වර්ගීකරණය කිරීම.

ප්‍රධාන පර්යේෂකගේ නම - වෛද්‍ය බුද්ධිණී සමරවීර

අධීක්ෂකතුමාගේ නම - වෛද්‍ය J. I. අබේනායක

වෛරස් පිළිබඳ විශේෂඥ වෛද්‍ය වෛද්‍ය පර්යේෂණ ආයතනය

කොළඹ 08

..... මහතා/මිය වන මම, මහරගම පිළිකා රෝහලේ මුඛය හා උගුර ආශ්‍රිත පිළිකා රෝගීන්ගේ ''හියුමන් පැපිලෝමා වයිරසය'' ඇත්දැයි සොයා බැලීම සඳහා වෛද්‍ය බුද්ධිණී සමරවීර විසින් සිදුකරනු ලබන පශ්චාත් උපාධි පුහුණුවේ කොටසක් වන මෙම පර්යේෂණය පිළිබඳව නිසි ආකාරයෙන් දැනුවත් වී ඇති අතර වෛරසය ඇත්දැයි සොයා බැලීමට මුඛය සෝදා ලබාගන්නා දියර සාම්පලය ගැනීමෙන් මට කිසිදු ආකාරයකින් හානියක් සිදු නොවන බවට දැනුවත් වීමි.

මෙම පර්යේෂණයෙන් ලබා ගන්නා තොරතුරු පර්යේෂණ කටයුතු සඳහා පමණක් යොදා ගන්නා අතර වෙනත් කිසිදු පාර්ශවයකට ලබා නොදී රහස්‍යබව රැකගන්නා අතර පර්යේෂණ ප්‍රතිඵල ප්‍රසිද්ධ කරන විට මාගේ අනන්‍යතාව හෙළි කරන බවට ද දැනුවත් වීමි.

මෙම පර්යේෂණයට සහභාගි වීමට එකඟ නොවීමට හෝ එකඟ වීමෙන් අනතුරුව පර්යේෂණයෙන් ඉවත්වීමට ද මා හට හැකියාව හා නිදහස ඇති බවට දැනුවත් වීමි.

මෙම පරීක්ෂණයට සහභාගි වීමට මාගේ කැමැත්ත ලබා දෙමි.

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රෝගියා රැකබලා ගන්නා

පුද්ගලයා/අයිතිකරු

....

සහභාගි වන්නාගේ පර්යේෂකයා දිනය

No : _____ Date: __/__/____

அனுமதிப் படிவம்.

தொடர் இலக்கம் :

திட்டத்தின் தலைப்பு : மகரகமவிலுள்ள புற்று நோய் நிறுவனத்தின் வாயும், வாய் தொண்டை தொடர்பான புற்று நோய் நோயாளர்களின் மனித பபிலோமா வைரஸ் ஜீனோடைபிங் ஐ கண்டு பிடித்தல்.

ஆராய்ச்சியாளரின் பெயர் : வைத்தியர். புத்தினி சமரவீர

மேற்பார்வையாளரின் பெயர் : வைத்தியர். ஜே. சுய். அபேனாயக்க.
வைரஸ் நுண்ணுயிரியல் ஆலோசகர்,
மருத்துவ ஆய்வுகூட நிலையம்,
கொழும்பு - 08.

நான் _____ "மகரகமவிலுள்ள புற்று நோய் நிறுவனத்தின் வாயும், வாய் தொண்டை தொடர்பான புற்று நோய் நோயாளர்களின் மனித பபிலோமா வைரஸ் ஜீனோடைபிங்ஐ கண்டு பிடித்தல்" எனும் கற்கைநெறி தொடர்பு அறிவுறுத்தப்பட்டது. இக்கற்கைநெறியானது பட்டப்படிப்பின் பின் பயிற்சி புரியும் வைத்தியர். புத்தினி சமரவீரனால் நடாத்தப்பட்டது. மனித பபிலோமா வைரஸை கண்டறிவதற்காக வாயிலிருந்து சுரண்டி எடுக்கப்படும் மாதிரியினால் ஏற்படும் நோவு, கஷ்டம் எதுவும் இல்லை எனவும் நான் தெளிவாக அறிவுறுத்தினேன்.
இக்கற்கைநெறி ஆய்வின் போது சேகரிக்கப்படும் தகவல்கள் ஆய்வுக்கு மாத்திரமே பயன்படும் எனவும் இவ்வாய்வின் முடிவுகள் பகிரங்கப்படுத்தப்படமாட்டாது எனவும் நான் விழிப்புடன் இருக்கிறேன்.

No: Date: __/__/____

இக்கற்பத்தவன். அனுமதிக்காக நான் முழு சுயபுத்தியுடனும் சுதந்திரத்துடனும் மகிழ்ச்சியுடன் உறுதி கூறுகிறேன்.

மேற்கூறப்பட்ட ஆய்வுப்பயிற்சிக்காக நான் பூரண சம்மதம்.

கவனிப்பு செய்பவர் / நோயாளியின் சொந்தமானவர்.

பங்கு பற்றுபவர் ஆராய்ச்சியாளர் திகதி.

ANNEXURE 3c

QUESTIONNAIRE

1. Serial No. -
2. Age (on date of data collection) -
3. Current occupation (monthly income) -
4. Residential area -
5. Educational level – Schooled/Not schooled

   If schooled, up to which level -
6. Gender –Male/Female
7. Marital status- Married/Unmarried

   If married-Age at marriage -
8. History of genital and oral warts- Yes/No
9. History of cancers – Yes/No

   If yes, type of cancer-
10. Past history of HPV vaccination -Yes/No

    If yes, when and how many doses
11. Alcohol consumption- Yes/No

    If yes-Units/day-
12. Smoking –Yes/No

    If yes- No. of cigarettes smoked/day-
13. Betel chewing –No/Yes

    If yes- No. of betel chewed / day -

ප්‍රධාන පර්යේෂකයා විසින් අසන ප්‍රශ්න

දිනය - .....

- රෝගියාගේ අංකය -
- වයස (දත්ත එකතු කරන දිනට) -
- වත්මන් රැකියාව (මාසික ආදායම) -
- ජීවත් වන ප්‍රදේශය -
- අධ්‍යාපන මට්ටම - පාසැල් ගොස් ඇත/නැත
    - ඇත්නම්, කුමන මට්ටම දක්වාද?
- ස්ත්‍රී / පුරුෂ
- විවාහක / අවිවාහක -
    - විවාහක නම් විවාහ වූ වයස -
- ලිංගික සහකරුවන් ගණන - එක් පුද්ගලයෙක් / එක් පුද්ගලයෙකුට වඩා වැඩි
- ප්‍රථම ලිංගික එක්වීම සිදු වූ වයස -
- පිළිකා තිබේද ? ඔව් / නැත
- මීට ප්‍රථම හියුමන් පැපිලෝමා වයිරයස සඳහා එන්නත් කර තිබේ ද? ඔව් / නැත
- මත්පැන් පානය කරනවාද ? ඔව් / නැත
    - ඔව් නම්, දිනකට පානය කරන ඒකක ගණන
- දුම් පානය කරනවාද? ඔව් / නැත
    - ඔව් නම්, දිනකට පානය කරන සිගරට් ගණන
- බුලත් හපනවාද? ඔව් / නැත
    - ඔව් නම්, දිනකට හපන බුලත්විට ගණන
        - පුවක්
        - දුම්කොළ
        - හුණු
- පිළිකා වර්ගය -
    - Stage –
    - හඳුනාගෙන කොතරම් කාලයක්ද?

நேர்காணல் முறை வினாத்தொகை.

தொடர்பு இல.

வயது (கின்னுடைய நிகதியில்)

தொழில் (மாத வருமானம்)

வசிக்கும் இடம்.

கல்வி அறிவு மட்டும் - (பாடசாலை சென்றார் / செல்லவில்லை).
சென்றார் ஆயின் கல்வி அறிவு மட்டம்

பால் - ஆண் / பெண்.

திருமண நிலை - திருமணமானவர் / திருமணம் ஆகவில்லை.

திருமணமானவரெனின் திருமணமான வயது

உங்களுக்கு வாயில் அல்லது பாலியல் உறுப்பு பகுதியில் (பால் உண்ணும் போட்ஸ்) ஏற்பட்டிருக்கிறதா? ஆம் / இல்லை.

புற்று நோய் உள்ளவரா / புற்றுநோய் ஏதாவது ஏற்பட்டதா? ஆம் / இல்லை
ஆம் ஆயின் என்ன வகையான புற்றுநோய்?

நீங்கள் HPV நோய்த்தடுப்பு மருந்து பெற்றுக்கொண்டீர்களா? ஆம் / இல்லை.
ஆம், ஆயின் எப்போது? எத்தனை முறை மருந்து ஏற்றிக்கொள்ளப்பட்டது

திருமணம் (மதுபானம் சாராயம் போன்றவை) பாவிப்பவரா? ஆம் / இல்லை.
ஆம் ஆயின் ~~வாரத்தில்~~ போதில் / நாள்.

புகைபிடித்தல் பழக்கமுள்ளவரா? ஆம் / இல்லை.
ஆம் ஆயின் நிகரட் எண்ணிக்கை / நாள்.

வெற்றிலை சீவும் பழக்கமுடையவரா? ஆம் / இல்லை.
ஆம் ஆயின் வெற்றிலை எண்ணிக்கை / நாள்.

ANNEXURE 4

DATA EXTRACTION SHEET

Serial No.	Positive/Negative For Human Papillomavirus	If positive, genotype

ANNEXURE 5a

Procedure for HPV DNA extraction from swabs

(Bioron-RealLine DNA – Express- VBC8899)

HPV DNA extraction steps

1. Determine the appropriate number of test tubes with Lysis Reagent needed for patient specimen and control testing.
2. Label tubes for each patient specimen and label additional tubes for NC and PC.
3. Spin briefly to collect all transport solution containing the test material from the walls of the tubes.
4. Resuspend cell pellet formed by centrifugation.
5. Add 100 µl of each specimen into a tube with Lysis Reagent using disposable tips with aerosol barrier.
6. Add 100 µl of Negative Control to the tube labeled NC.
7. Add 100 µl of Positive Control to the tube labeled PC.
8. Close the tubes securely.
9. Vortex thoroughly for 10 sec.
10. Place the tubes into a heating block and incubate for 15 minutes at 98 °C.
11. After heating cool the tubes down to room temperature, then centrifuge at 8000 rpm at room temperature for 1 min.
12. Use the resulting supernatant as a DNA-containing sample suitable for further PCR analysis.

Important notes

- Store the kit at (2-8) °C in the manufacturer's packing.

- Isolated DNA specimens can be stored at (2-8) °C for no longer than 24 hours.
- If samples of isolated DNA were stored frozen prior the assay, thaw them and keep at least 30 minutes at a temperature of 18 to 25 °C.

References

1. BIORON Diagnostics GmbH RealLine DNA – Express VBC8899- Instructions for Use

ANNEXURE 5b

HPV DNA Extraction

Extraction done by : _____ Date : _____

Name of kit: _____ Lot No: _____ Expiry date: _____

No of samples: _____

Lysis reagents Lot No: _____ Exp date : _____

ANNEXURE 6a

Procedure for HPV Genotyping Real time PCR

(Bioron-RealLine HPV High Risk Genotype (Fla-Format)-VBD8482)

Preparation of the working reagents

- Prior the tests take the kit out of the refrigerator and keep the Master Mix (MM 1, 2, 3, 4) closed in the package at 18 - 25 °C for at least 30 minutes.

Test-tube	HPV types
MM1	16, 18, 39
MM2	33, 45, 56
MM3	31, 35, 58
MM4	52, 51, 59

- Take the necessary number of tubes with MM (including prepared samples and controls: 1 Negative control-NC and 1 Positive control-PC). Each sample and control needs to be analyzed using 4 MM -MM 1, 2, 3, 4.
- To prepare diluted Master Mix, add 250 µl of Recovery Solution (RS) to each tube with MM.
- Mix gently, holds at room temperature for 15 minutes and then carefully re-mixes.
- Store diluted MM at 2 to 8 °C for 2 weeks.

PCR procedure

- Prepare an appropriate number of 0.2 ml tubes. Label each tube for each specimen and controls
- Need 4 tubes each (separately for MM1-4) for PC, NC and NTC (No template control) and samples.
- Add 25 µl of diluted Master Mix to each 0.2 ml tube.

ANNEXURE 6a

- Add 25 µl of corresponding isolated DNA solution to each tube using a separate pipette tip with filter (each sample add to 4 tubes containing MM 1, 2, 3, 4). Add 25 µl of PCR grade water to tube labeled NTC.
- Do not touch the pellet.
- Tightly close the tubes and give a short spin.
- Place the tubes into the Real Time PCR system.
- Program Real Time PCR system.

Settings for ABI 7300 and ABI 7500 Cycler

- Define the following settings

Settings	
Reaction volume	50 µl
Ramp Rate	Default
Passive reference	None

- Define the fluorescent detectors (dyes):

Target	Detector
Types 16,33,31,52	FAM
Types 39, 56, 58, 59	JOE
Types 18, 45, 35, 51	ROX
Internal control MM1-4	Cy5

ANNEXURE 6a

- Define the temperature profile and dye acquisition:

Program name	Cycles	Target (°C)	Acquisition mode	hold
Pre-incubation UDG "carry over protection"	1	50	none	2 min
	1	95	none	2 min
Amplification	45	94	none	10 s
		60*	yes	35 s

*Fluorescent signal detection

Data Analysis and interpretation

- Use the automatic baseline and threshold (Auto Ct).
- For the positive controls of MM1-4, Ct values for FAM, JOE, and ROX should be ≤35.
- For the negative controls of MM1-4, Ct values for FAM, JOE, ROX should be >35 and Cy 5 should be in the accepted range*.
- For each sample in MM 1 - 3 an increase of the amplification signal of IC DNA (channel Cy5) should be detected and IC Ct determined*.

***Calculate (IC Ct)av as an average IC Ct of all analyzed samples (including PC and NC) independently for each of MM 1-3. IC Ct values that differ by more than 2 cycles from the (IC Ct)av should be ignored. Recalculate the (IC Ct) av for the remaining values after the screening.**

- For each sample in MM 4 an increase in human β-actin DNA amplification signal (channel Cy5) should be recorded.
- The sample is considered valid if the Ct value for this sample via Cy5 in MM 4 is less than or equal to 32.

ANNEXURE 6a

- For samples found to be invalid (Ct through the Cy5 channel in MM4 is above 32), a repeated collection of specimens is required.
- The sample is considered positive, i.e. contains *HPV DNA*, when Ct value via FAM JOE and ROX channels for this sample (in any of MM 1 - 4 tubes) is less than or equals to 35.
- The sample is considered negative (not containing HPV DNA of the corresponding type), if Ct value via FAM JOE and ROX channels for this sample is above 35 or is not determined.

Table 3

	Positive detection signal in the channel		
Tube	**FAM** *Green*	**JOE** *Yellow*	**ROX** *Orange*
MM1	type 16	type 39	type 18
MM2	type 33	type 56	type 45
MM3	type 31	type 58	type 35
MM4	type 52	type 59	type 51

References

BIORON Diagnostics GmbH RealLine HPV High Risk Genotype-Fla-Format- VBD8482

ANNEXURE 6b

HPV Genotyping Real time- PCR

25 µl MM+25 µl DNA extract

Assay run by- _____ **Date-** _____

Kit- _____ **Cat # -** _____ **Lot # -** _____ **Exp Date -** _____

Master Mix type - _____ Opened on - _____ Exp Date - _____

Positive Control type -_____ Opened on - _____ Exp Date - _____

1 PC MM-1	**9** Sample 1 MM-1	**17**	**25**	**33**	**41**
2 PC MM-2	**10** Sample 1 MM-2	**18**	**26**	**34**	**42**
3 PC MM-3	**11** Sample 1 MM-3	**19**	**27**	**35**	**43**
4 PC MM-4	**12** Sample 1 MM-4	**20**	**28**	**36**	**44**
5 NC MM-1	**13**	**21**	**29**	**37**	**45** NTC MM-1
6 NC MM-2	**14**	**22**	**30**	**38**	**46** NTC MM-2
7 NC MM-3	**15**	**23**	**31**	**39**	**47** NTC MM-3
8 NC MM-4	**16**	**24**	**32**	**40**	**48** NTC MM-4

Validation

PC – MM-1_____MM-2 _____MM-3 _____ MM-4 _____

NC- MM-1 _____ MM-2 _____ MM-3 _____ MM-4 _____

ANNEXURE 6b

NTC- MM-1 _____MM-2 _____ MM-3 _____ MM-4 _____

Samples – IC-MM-1 _____ MM-2 _____ MM-3 _____ MM-4 _____

Checked by- _____ Authorized by- _____ Date- _____

Ethics Review Committee

Medical Research Institute

17.06.2016

Dr. Buddhini Samaraweera,
MD Trainee in Medical Virology

Project No: 27/2015

Detection and genotyping of human Papillomavirus in patients with oral and oropharyngeal carcinoma at the Cancer Institute, Maharagama.

The above project was reviewed for Scientific and ethical aspects and approved by the Ethics Review Committee of the Medical Research Institute at a meeting held on the 11th June, 2015.

You are expected to inform the following to the Research and Ethics Committees.

1. Any deviations that you are planning to carry out during the conduct of the research for prior approval
2. Progress reports at 3 month intervals
3. Final report prior to the publication

.....
Dr. Rajiva de Silva
Chairperson

Copies – File E/C
Accountant / MRI (for information)

Medical Research Institute,
Danister de Silva Mawatha,
P.O. Box 527, Colombo 08

Phone: 011 2696234 E mail :rc.ec.mri@gmail.com
Fax :011 2691465 Web: http://www.mri.gov.lk

Approved

Dr. G. WIJESURIYA
MBBS, DTCD, MSc (Med. Ad.)
Msc (Food & Nutrition)
DIRECTOR
National Cancer Institute
Maharagama

25/07/2015

The Director,

Cancer Institute-Maharagama.

Dear sir,

permission to collect samples for project-27/2015

I am Dr.Buddhini Samaraweera, PG trainee in Medical Virology at MRI have to carry out a dissertation as a part of my MD training course. I selected **Detection and genotyping of human Papillomavirus in patients with oral and oropharyngeal carcinoma at the Cancer Institute,Maharagama** which will be starting from 15th of December 2015 . Here with I attached my research proposal and the permission letters from MRI Research committee for your further information.

Please be kind enough to grant the permission to collect samples from wards_____ and [illegible]

Thank you.

Principal Investigator,

Dr.Buddhini Samaraweera,

MD Trainee in Medical Virology.

Supervisor- Dr.J.I.Abeynayake,

Consultant Virologist,MRI

My No.AAJ/ETH/COM/2015

The National Hospital of Sri Lanka,
Colombo.

17.12.2015

Dr. Buddhini Samaraweera
PG trainee in Medical Virology at MRI.

REQUEST FOR A STUDY ON " DETECTION AND GENOTYPING OF HUMAN PAPILLOMAVIRUS IN PATIENTS WITH ORAL AND OROPHARYNGEAL CARCINOMA AT THE CANCER INSTITUTE, MAHARAGAMA"

Reference your letter dated 10th December, 2015 on the above subject , I wish to inform you that the permission has been granted by the Ethical Committee of this hospital for your study after resubmitting the questionnaire in three languages.

Chairman
Ethical Review Committee

Deputy Director (OPD Services)
National Hospital of Sri Lanka
Colombo 10.

Applied Biosystems 7300/7500

Settings for ABI 7300 and ABI 7500 Cycler

Recommended RealLine Pathogen Formats: **Fla-format (Flask)**
To the tube with the mastermix the Recovery Solution has to be pipetted, please read the IFU for volumes. After incubation at room temperature the mix has to be pipetted to tubes or plates and the same amount of DNA/RNA solution has to be added.

We can recommend to use: 10 µl DNA + 10 µl Mastermix
or as described in the IFU: 25 µl DNA +25 µl Mastermix

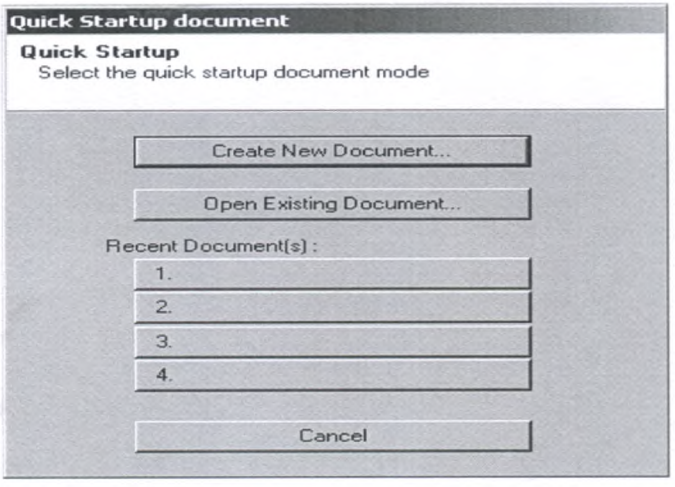
For **Str-format**: 50 µl DNA/RNA solution has to be pipetted directly to the lyophilized mastermix in the tubes, carefully mixed and distributed to a suitable plate or tubes.

Please read the Instruction manual of the kit for further informations.

I. CREATE A NEW DOCUMENT

1. Select **Start > All Programs > Applied Biosystems > 7300/7500 System > 7300/7500 System Software** to start the SDS software.

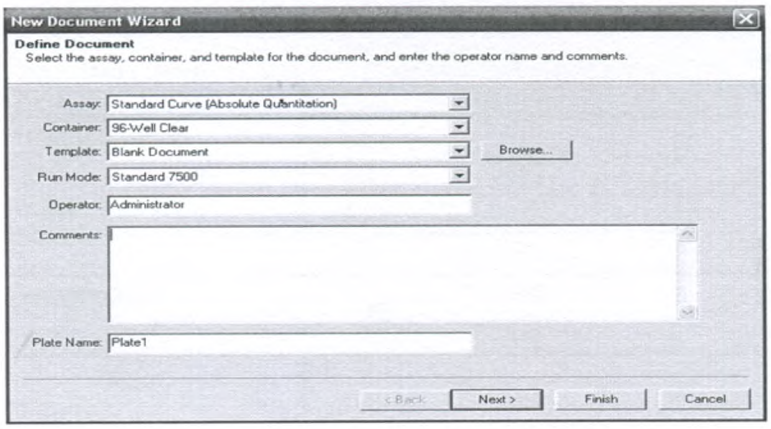
2. In the Quick Startup document dialog box, select **Create New Document**.



3. In the Assay drop-down list of the New Document Wizard, select **Standard Curve (Absolute Quantitation)**.

BIORON Diagnostics GmbH- Rheinhorststraße 18- 67071 Ludwigshafen (Germany)
Phone: +49 621 545 900 70 Fax: +49 621 545 900 68
E-Mail: info@bioron.de Homepage: www.bioron.de

4. Enter a name in the Plate Name field.



5. Select detectors according to the data sheets of the kits:

- **FAM**
- **ROX**
  And in multiplexing kits
- **VIC/JOE (HEX)**

6. Specify the detectors and tasks for each well. **No passive Dye!**

7. Enter the sample names.

8. Verify the information on each well in the Setup tab.

Applied Biosystems 7300/7500

II. TEMPERATURE PROFILES

1. For the detection of **DNA**: create a temperature profile on your instrument as follows:

Program name	Cycles	Target (°C)	Acquisition mode	hold
Pre-incubation UDG "carry over protection"	1	50	none	2 min
	1	95	none	2 min
Amplification	45	94	none	10 s
		60*	yes	35 s

*Fluorescent signal detection

2. For the detection of **RNA**: create a temperature profile on your instrument as follows:

Program name	Cycles	Target (°C)	Acquisition mode	hold
Reverse transcription	1	45	none	30 min
	1	95	none	2 min
Amplification	45	94	none	10 s
		60*	yes	35 s

*Fluorescent signal detection

Note: Kits for the detection of RNA and DNA can be run in parallel in one run. Choose the settings for the detection of RNA p. II.2

Specify thermal cycling conditions and start the run:

- Sample Volume is in the range of 10 to 50 µl
- Verify desired Run Mode.

- Select **File** > **Save**, enter a name for the plate document, then click **Save**.
  (Optional) If you want to use this settings again save it as a template document. Select **File > Save As**. In the **Save in** drop-down list,
  Navigate to **Applied Biosystems\7300\7500\7500\Templates**. Type the File name, then select (*.sdt) for **Save as type** to save the file as a template.

- Load the plate into the instrument

- Click Start.

III. ANALYSIS

Configuring Analysis Settings

Use the automatic baseline and threshold feature of the SDS software (**Auto Ct**). If the baseline and threshold were called correctly for each well, you can proceed to view the results.
The SDS software automatically generates baseline values for each well and threshold values for each detector.

IV. RESULTS

Please see the result analysis interpretation in the RealLine protocols.
The Ct value of the Internal Control IC is in the range of 27-31 depending on the plastic ware and other parameter.

Note: This settings are a guidance with recommendations.
BIORON Diagnostics GmbH can not be held liable for the use of this content.

Annexure 11

TEST METHOD VERIFCATION SUMMARY SHEET

Assay: HPV Genotyping PCR, Qualitative Assay
RealLine HPV High Risk Genotype Fla-Format
BIORON Diagnostics GmbH
Lot No - 16

FDA approval	-	IVD /CE	√
Existing kit	-	New Kit	√
Quantitative	-	Qualitative	√

Method: Real-time PCR

Instrument: ABI 7500

Date Range of Verification: 15/12/2015 – 18/12/2015

Sample Types Tested: Reference specimens of HPV 16 and 18
Total 20
HPV 16 DNA positive specimens: 5
HPV 18 DNA positive specimens: 5
HPV 16, 18 DNA negative specimens : 10

Comparison with:
Expected results

Sensitivity:
HPV 16:100% (5/5)

HPV 18:100% (5/5)

Specificity:
100% (10/10)

Additional Comments:

Sensitivity and specificity of other genotypes (31, 33, 35, 39, 45, 51, 52, 56, 58, and 59) were not tested due to the limited resources

Verification and summary completed by: Dr. B Samaraweera **Date:** 18/12/2015

The verification study has been reviewed and the performance of the method is considered acceptable for patient testing.

Consultant Medical Virologist _____ **Date:** 18/12/2015