

## SALIVARY HUMAN PAPILLOMAVIRUS INFECTION IN HEALTHY PEOPLE

Virna-Maria Tsitou<sup>1✉</sup>, Dimitrios Rallis<sup>2</sup>, Mariana Tsekova<sup>3</sup>,  
Nikolay Yanev<sup>4</sup>

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### Abstract

In recent years, interest in human papillomavirus infections as a causative factor in epithelial cancer development has grown. Literature indicates that HPV involvement in malignant transformations in oral mucosa can vary significantly, from 0 up to 87%. The aim of our study was to detect the prevalence of salivary HPV infection among generally healthy adults. The examination involved 139 patients, from whom 139 whole, 1.5 ml saliva samples were obtained. HPV DNA was detected by the nested PCR technique. To visualize the PCR products electrophoresis reactions were carried out. Sample analysis showed that DNA for HPV was detected in 14 patients: 11 positive results were obtained from men, and 3 from women. This yields a high infection rate: 10.07%. The HPV prevalence in the male group was more than twice as high as in the female group. Also, subclinical oral HPV infection was detected more frequently in young (19–39 years old) and older ( $\geq 60$  years old) adults.

**Key words:** human papillomavirus, saliva, healthy adults

**Abbreviations:** CTAB – cetyltrimethylammonium bromide, EDTA – ethylenediamine tetraacetic acid, HPV – human papillomavirus, OLP – oral lichen planus, PCR – polymerase chain reaction, SCC – squamous cell carcinoma, TAE – Tris-acetate-EDTA

**Introduction.** In recent years interest has grown in human papillomavirus infections as a causative factor in epithelial cancer development [1–5]. Human papillomavirus is part of a larger DNA virus family, the Papillomaviridae. They are built up from double-stranded DNA. So far more than 100 HPV genotypes have

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been described. They are divided into two groups: high and low oncogenic risk. Among low-risk HPVs we can distinguish, e.g., HPV6, HPV11, HPV1, HPV2. High-risk HPVs such as HPV16, HPV18, HPV31, HPV33, HPV45 are described as having high potential for malignant progression [4]. Almost 45 genotypes of HPV have been detected in cervical cancer 1. The high oncogenic potential of some HPV genotypes is believed to relate to two specific oncoproteins: oncoproteins E6 and E7, which are crucial for promoting viral transformation in epithelial tissue. Those two oncoproteins alter the function of genes p53 and pRb, which are responsible for tumour suppression. Both of those oncoproteins trigger proliferation and cell immortalization and induce genomic instability [4].

Sound skin or oral mucosa is resistant to its inoculation. Healthy individuals can be reservoirs for HPV. The incidence of HPV in not pathologically changed oral epithelium can vary from 0.6% up to 81%. This discrepancy is due to the DNA detection methods used, and the means of specimen acquisition. The oral cavity can be infected with HPV by autoinfection, during labour from an infected mother to a child, or by oral sex [3, 6, 7]. No evidence exists that HPV is an airborne virus.

Human papillomavirus as a cervical cancer factor has been widely proven, both biologically and epidemiologically [4]. Literature indicates that HPV involvement in malignant transformations in oral mucosa can range from 0 up to 87%. HPV16 considered a high-risk genotype is the most prevalent HPV type in cervical squamous cell carcinoma; it is also the most detected HPV type in all head and neck SCC [1, 3, 5–8].

The aim of our study was to detect the prevalence of salivary HPV infection among generally healthy adults.

**Materials and methods. Patients.** The examination was performed on 139 patients in good general condition: university students, hospital staff, and members of the general public, without oral mucosal lesions. The oral cavity state was checked in a clinical examination. Subsequently after about fifteen minutes of rest, unstimulated saliva samples were collected into an Eppendorf tube by a dentist. All research participants gave written consent to take part in the study.

To provide full anonymity each of the patients was given an individual identification number.

**DNA extraction.** Saliva was collected by spitting into a sterile 1.5 ml Eppendorf tube; samples were stored at  $-24^{\circ}\text{C}$  until testing. Before molecular biology analysis, obtained saliva samples were thawed on ice. DNA isolation was performed, using a modified cetyltrimethylammonium bromide (CTAB) protocol optimized for salivary DNA extraction. This approach is based on improvements described in recent literature for isolating high-yield nucleic acids from human oral samples [9]. The quality of the extracted DNA was verified using  $\beta$ -globin PCR prior to viral DNA detection. As an internal control of the DNA extraction, samples with DNA were subjected to end-point PCR for human  $\beta$ -globin gene

amplification, by using GH20 5'(GAAGAGCCAAGGACAGGTAC)3' and PCO4 (5'TCACCACAACCTTCATCCACGTTCCACC3') oligonucleotides, which flank a sequence of about 268 pb. Samples that were positive for human  $\beta$ -globin gene were subjected to viral DNA search for HPV. After reanalysing samples for confirmation only negative samples for  $\beta$ -globin gene were excluded from the analysis.

**HPV DNA amplification.** In our study HPVs were detected by nested PCR. This PCR required two different, conventional pairs of oligonucleotide primers to detect HPV: MY09/MY11, GP5+/GP6+, one by one. The PCR reaction mixture was prepared according to well-defined proportions of reagents. A sample calculated per one reaction was: 25  $\mu$ L DreamTaq Green PCR MasterMix (Thermo Scientific), 18.75  $\mu$ L Water nuclease free (Thermo Scientific), 1.25  $\mu$ L of primers at a concentration of 20 pml/ $\mu$ L and 5  $\mu$ L suspension of isolated DNA from saliva. For each series of samples subjected to nested PCR, a positive control sample and also a negative control sample (H<sub>2</sub>O) were carried out. Positive control material contained purified genomic HPV type 16 DNA suspension: Amplirun Papillomavirus DNA Control Sample (Vircell Microbiologists) at concentration 1000 copies/of genomic HPV DNA. The temperature and conditions of nested PCR were adjusted to the recommendations of the manufacturer's Dream Taq Green PCR Master Mix and the melting temperatures of the primers (Table 1). The amplified genetic material was subjected to electrophoresis for several hours from the time of isolation, and until then kept in a refrigerator at 4 °C.

**Electrophoresis reaction.** To visualize the PCR products, an electrophoresis reaction was carried out in a 2% agarose gel in the 1 $\times$  concentrated TAE mixture (242 g Tris pH 8.3; 100 ml 0.5 M EDTA pH 8; 57.2 ml CH<sub>3</sub>COOH per 1000 ml) in the presence of MIDORI Green Advance DNA Stain (NIPPON Genetics EU-

T a b l e 1

Conditioning protocol of nested PCR technique using primer pairs: MY09/11 and GP5+/6+

Set primers	Reaction step	Temperature, °C	Duration	Number of repetitions
MY09/11	Initial denaturation	95	3 min	—
	Denaturation	95	30 s	—
	Hybridization of primers	53	30 s	40 $\times$
	Amplification DNA	72	45 s	—
	Final amplification	2	5 min	—
	—	—	4	$\infty$
GP5+/GP6+	Initial denaturation	94	5 min	—
	Denaturation	94	1 min	—
	Hybridization of primers	48	2 min	30 $\times$
	Amplification DNA	72	1 min	—
	Final amplification	72	5 min	—
	—	—	4	$\infty$

ROPE GmbH). For the number of base pairs, a standard DNA GeneRuler 100 bp DNA Ladder (Fermentas, Thermo) was used. Visualization of electrophoresis results was performed under UV light with the use of Gel DOC EZ Imager, BioRad.

**Results.** The research material consisted of 139 saliva samples, from 139 patients: 57 women and 82 men, aged between 19 and 74 years. Sample analysis showed that DNA-HPV was detected in 14 patients: 11 positive results were obtained from men, and three from women. Mean patient age was 32. The study revealed that the most common HPV DNA was detected in subjects between 19 and 39 years old. Genetic material of the virus was detected in 12 samples from subjects in this age range (Table 2).

T a b l e 2

Total sample description and detection of DNA-HPV in relation with gender and age groups

	Patients <i>n</i> = 139	Positive HPV result <i>n</i> = 14
<b>Male</b>	82	11
<b>Female</b>	57	3
<b>19–39 years old</b>	106	12
<b>49–59 years old</b>	9	0
<b>60+ years old</b>	24	2

**Discussion.** Numerous studies have assessed the prevalence of human papillomavirus in the oral cavity. HPV was detected in various oral specimens collected from saliva, oral rinse samples, gargle with mouthwash samples, epithelial cells after exfoliation, from different pathological oral lesions, and even from healthy, asymptomatic oral mucosa. In these studies the occurrence of the oral HPV infection was sometimes completely different, ranging from 1.9% up to 10% [10–12]. This could be explained by the use of other DNA extraction methods, different populations with low or high risk for the infection, populations from different geographical regions, and of course because of different sample collections [13–17].

In our study collection of 1.5 ml of whole saliva took about 7 min in patients with proper salivary flow. In order to check the frequency of asymptomatic HPV infections, 139 healthy people were qualified for the study – e.g., without immunodeficiency, not organ recipients, not during immunosuppressive therapy. This is due to the fact that the frequency of infections, including HPV, among people with a disturbed immune system could be significantly higher than in healthy people of the same age. The average patient’s age was 32 years. We estimated general HPV infection for all HPV types.

Fourteen samples out of 139 revealed positive results for HPV: this is a high infection rate, at the level of 10.07%. A bit lower levels of HPV infection were presented by other researchers for all types of HPV in healthy individuals: 7.7% in the meta-analysis of 66 studies done by TAM et al. [10], 7.5% in the work of WOOD et

al. [11] in which 3762 individuals were included. However, a lower frequency (6.9%) was demonstrated in the work of GILLISON et al. [12] with 5579 participants, and prevalence of 5.5% in the meta-analysis of SHIGEISHI and SUGIYAMA [13] of 29 studies with 22 756 individuals. A case-control study conducted in England by HEARDEN et al. [14] in a mixed population of 700 participants, incorporating university students, hospital staff, dental patients, and the general public, the prevalence of oral HPV was 5.5%, while in a meta-analysis of 48 reports comprising 28 544 subjects MENA et al. [15] reported even lower infection frequency, at 4.9%. In our research group there were 59% males and 41% females. It is worth underlining that HPV prevalence in the male group was more than twice as high (13.41%) as in the female group (5.26%).

Similar observations have been presented in other studies. CHATURVEDI et al. [16], on the basis of a large representative population of 11 million men and 3.2 million women, found that the prevalence of HPV infection was three times more frequent in males than in females: 11.5% and 3.2% accordingly. Similarly on the basis of a USA cross-sectional study of a large number of participants (5579), Gillison et al. [12] revealed three times higher HPV infection prevalence in men (10.1%) when compared to in women (3.6%).

The group of people between 19 to 39 years old constituted 76.26% of all of the participants; HPV infection was detected in 11.32% of them, the highest rate.

The increase in HPV infections in the age group 19–39 could be related to the increased sexual activity of individual people in this group.

None of the subjects in the middle aged group (40–59 years old), 6.67% of the entire group of patients, had the HPV infection. In turn, senior patients ( $\geq 60$  years old), 17.26% of all of the study subjects, revealed HPV infection at the level of 8.33%.

Similar findings were also demonstrated by other authors, where the HPV infection prevalence peak was observed among young people aged 30 to 34 years old (7.3% infected), and elders 60–64 years old (11.4%) [10].

On the one hand, our findings and those of others prove the frequent presence of HPV infections in the oral cavity, which can be mostly asymptomatic. On the other hand, mucosal lesions related to HPV infections are observed as focal epithelial hyperplasia, oral papillomas, oral warts [18]. These pathological lesions are not common and have no carcinogenic potential, as they are related to low-risk HPV types such as HPV (6,13,32).

The occurrence of HPV infection in oral mucosal lesions is also not clear. DE ABREU et al. [18] investigated the status of HPV in a cohort of 90 biopsied oral squamous cell carcinomas (OSCC) of Brazilian patients. Study showed a low 3.3% frequency of HPV infection and detection of HPV16 in all of the cases, which is the most common type. Additionally, this study revealed that in pre-malignant non-homogeneous leukoplakias HPV was found less frequently than in homogenous types, which are considered to have better prognosis due to lower levels of malig-

nancy. However, oral leukoplakia shows an increased risk of HPV infection when compared to clinically healthy mucosa, with its prevalence of around 20% [19].

Finally, it must be emphasized that there are many risk factors contributing to oral malignancy development. HPV infection may not be the most important one but may be overlapping or inducing the other local and general factors. It is well known that HPV is the most common sexually transmitted infection. Its transmission to the oral cavity, and the enhancement of the risk of premalignancy and development of oral cancer, is increased in women with cervical infection that confirms the possible transmission between oral cavity and genitals.

Oral HPV infection is significantly higher, more than 10%, in women with cervical infection than in the general population of healthy women without cervical infection.

The study of COSSELLU et al. [6] reports the presence of oral infection in 20.4% of women with gynaecological infection. This study has limitations, because there are no additional parameters that could be taken into consideration when the assessment of the risk factors for HPV infection is conducted.

**Conclusions.** In our research the prevalence of HPV infection detected in whole saliva was a little higher than in studies conducted by cited authors. This study showed that subclinical oral HPV infection is present more often in men than in women. It was also more frequently seen in the young and in elder people. Our results propose that dental practitioners should have the knowledge of HPV risks and involve themselves in the early detection of all the oral mucosal lesions associated with HPV infection.

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<sup>1</sup>*Department of Medical Microbiology, Faculty of Medicine, Medical University of Sofia, 2 Zdrave St, 1431 Sofia, Bulgaria*  
 e-mails: maria-tsitou@hotmail.co.uk

<sup>2</sup>*El Greco Private Dental Care, 86 Vitosha Blvd, 1463 Sofia, Bulgaria*  
 e-mail: Dimirall85@gmail.com

<sup>3</sup>*Department of Imaging and Oral Diagnostics, Faculty of Dental Medicine, Medical University of Sofia, 1 G. Sofijski St, 1431 Sofia, Bulgaria*  
 e-mail: mpcekova@abv.bg

<sup>4</sup>*Yanev Medico-Dent Clinic, 37 Ami Boué St, 1612 Sofia, Bulgaria*  
 e-mail: nyanev@abv.bg