DETECTION OF HUMAN PAPILLOMAVIRUS 16 AND 18 DNA FROM ORAL EPITHELIAL CELLS BY THE REAL TIME PCR IN NORMAL ORAL CAVITY IN QUETTA

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ABSTRACT

Background: Human papillomavirus (HPV) are small DNA tumor viruses that play a great part in a number of benign and malignant proliferative diseases. The presence of the virus is often associated with the proliferation of benign and malignant neoplasms of the oral cavity and genital area. Among the HPV linked malignancies, HPV16 is prevailed higher than HPV18 in Oral Squamous Cell Carcinomas and in Oropharyngeal Carcinoma. The purpose of this cross sectional study was to investigate the prevalence of high risk HPV 16 and 18, and the association with its risk factors among Dental Patients with healthy oral mucosa.

Methods: Two hundred patients with normal oral cavity visited the Dental Section, Sandeman Provincial Hospital, Quetta, were engaged in this study. After interview the buccal epithelial exfoliated cells were collected by soft bristle tooth brushing and processed to DNA extraction.

Results: One hundred and ninety two PCR- positive out of 200 DNA samples further processed for the β-globin gene and these were subsequently examined for the presence of HPV 16 and 18 DNA by the Real-Time PCR. A total of 16 subjects (8.3%) harbored viral DNA, these included (4%) and (11%) positive samples for HPV16 and HPV18, respectively. One sample contained both HPV16 and HPV18. To analyze the all clinical parameters smoking usage was associated with the presence of HPV ($p=0.001$) while others clinical parameters were not significance.

Conclusion: In conclusion, the existence of HPV 16 and 18 DNA in normal oral epithelial cells appears to be low. This suggests oral cavity may serve as a reservoir of such viruses, especially in smoking subjects. However, a larger population may be required to investigate influences of other clinical parameters on HPV.

Key words: Human papilllomavirus, normal oral mucosa, real-time PCR

INTRODUCTION

Human papillomavirus HPV are small DNA tumor viruses which play a part in a number of proliferative diseases, and majority of them are responsible and knows as low and high risk HPVs and their association with benign and malignant proliferation throughout body like skin, oral cavity and genital areas. Both man and women can have the cancer of oral cavity and genital area by this DNA viruses[1]. (HPV) infection mostly transmitted sexually in both male and female observed worldwide. The presence of the virus is often associated with the proliferation of benign and malignant neoplasms of the oral cavity and genital area[2].

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Oral Squamous cell carcinoma is reported in the different part of the world and ranked eigth with regional epidemiologic variations but in south central Asia Oral Squamous Cell Carcinomas (OSCC) is ranked third. The World Health Organization believe that the number of (OSCCs) will increase worldwide in the next decades. In Pakistan due to frequent usage of smokeless tobacco like snuff, betel quid, the OSCCs are getting common and according to the survey, the OSCCs are the second most common of all malignancies in both male and female with the highest incidence among oral cancers in the world.

The prevalence of HPV 16 and 18 is debatable including in the Oral cavity. The percentage of HPV is different among the published data from 0-100%, dependently on degree of diseases, tissue origin, sampling size and management, detection techniques as well as ecological risk factors such as geological regions and high-risk behaviors. Among the HPV linked malignancies, HPV16 has higher prevalence than HPV18 in OSCCs and in Oropharyngeal Carcinoma[3]. A study conducted by Ali et al., reported that 90% out of 68% of the HPV associated in OSCCs from cheek and tongue were HPV16 positive cases[4]. Such high incidence is believed to be directly related to risk behaviors such as smoking, use of smokeless tobacco and alcohol consumption[5]. Another study conducted

in eastern India showed that 33.6% of the HPV positive cases among the OSCCs were addicted to tobacco[6].

HPV starts its life cycle by spreading to the basal cells of epithelium. Within these, viral reproductions often lead the development of virus related pathology or symptoms The identification of HPV in persons 'at risk' for HPV related disease might be an informative prognostic tool. The low risk types of HPV may be not effect the nature of life in the development of skin and genital and oral warts but also the unusual relationship betwerisk HPV and malignancy indicate the significance of early diagnosis when it is suspected that a lesion may harbor such HPV[7].

There have been few studies conducted regarding the presence of high risk HPV like 16 AND 18 in normal oral cavity without any lesions in Pakistani popultion. This study was conducted to determine the prevalence of types of high risk HPV 16 and 18 in normal oral cavity without any lesions or symptoms and its association to risk factorswhich may leed to a HPV prevention and treatment program.

MATERIAL AND METHODS

This was a crossectional study which included 200 patients through non probability consecutive sampling. The patients who visited the Dental Section of Sandeman Provincial Hospital, Quett for routine oral examination and agreed to participate in this study were included in the study. Each patient was informed about the protocol of research and signed the informed consent form. All volunteers were between 18-60 years old with no serious systemic diseases, and congenital diseases. All sociodemographic parameters like Age, Sex, Smoking and betel quid habits were noted.

Samples collection and DNA preparation from Oral epithelial cells

Oral epithelial cell were collected used by super soft bristle toothbrush from all mucosal area like buccal mucosa, palate and dorsum tongue dropping in 4 ml of a lysis buffer containing 0.5% sodium dodecyl sulfate, 1 mM EDTA, and 10 mM Tris. DNA from 600 μl of each sample was prepared using the QIAamp DNA Mini Kit (Qiagen® GmbH, Hilden, Germany) according to the manufacturer protocol. The extracted DNA was eluted in 200 µl of the eluting buffer (provided by the kit) and kept at -20°C until further use. The concentration and purity of the prepared DNA were spectrophotometrically measured at 260 nm and 280 nm (GENESYS 10uv, model 10-S USA).

Real Time PCR to investigate the presence of HPV

Real Time PCR was used to investigate the presence of HPV in this study, The results were showed by Real Time PCR in duplicate using the KAPA SYBR FAST qPCR Kit (Kapabiosystems®, MA, U.S.A) and the primers shown in Table 1. The employed primers showed no cross-reactivity with other sequences of other organisms in the GenBank using the BLAST search. The β-globin primers, KM 29 and KM 138, were employed to confirm successful DNA extraction from each sample. All β-globin-positive samples were subjected to real-time PCR using the set of HPV 16 and HPV18 primers. Each Real-Time PCR mixture was prepared in a total volume of 10 µl containing 0.8 µl of each 2.5 µM of either primer pairs, 5 µl of 2X Master mix, 1 µl of investigated DNA and 2.4 µl of sterile distilled water.

The Real-Time PCR products were verified by two methods in this study including agarose gel electrophoresis and the Bio-Rad PrecisionMelt Analysis software, which was provided with the Bio-Rad iCycler®, U.S.A. After the PCR, the melting curve was obtained by melting each amplified product at a transition rate of 0.2°C for 10s from 70°C to 90°C. By gel electrophoresis, the amplicons were run on 1.5 % agarose gel electrophoresis at 80 volt for 30 min after that the agarose gel was stained in 0.5 ug/ml ethidium bromide solutions and analyzed under the UV transilluminator (Geldoc, Biorad®, CA, USA).

The collected data was analyzed using the SPSS version 17.0. The Socio demographic factors were noted and explain using the descriptive statistics such as number, percentages and means. The Pearson χ^2 test was employed to determine an association between the independent variables and HPV. The p-Value of less than 0.05 was considered significantly different.

RESULTS

Patient characteristics

One hundred and ninety two patients were β-globin positive out of two hundred. Majority of respondent were male (62.5%) and the average age was 40 years. Data showed that the majority of the respondents were non-smokers mean (53.1%) and (27.6 %) were old smokers and 37 (19.3%) were current smokers. In this study the majority of the respondents were smokeless tobacco users 111 (57.8) whereas 43 (22.4 %) were old and 38 (19.8%), were new users.

Detection of HPV16 and 18 from oral mucosa by Real Time PCR

In this study sixteen 16 (8.3%), out of 192 samples were positive for either HPV 16 or HPV 18. Four samples contained HPV16. The shape of the melt curves among the HPV16 positive samples, the amplicons ranged from, 72.2-72.8°C (Figure 1a). Where as 11 samples carried HPV18. The melting curve among HPV 18, amplicon ranged was 78.6-78.8°C, respectively (Figure 1b). Out of 16, one sample harbored both strains HPV16 and 18. The amplicon size 262-bp was obtained by agrose gel from the β-globin positive samples (Figure 2a). Similarly,

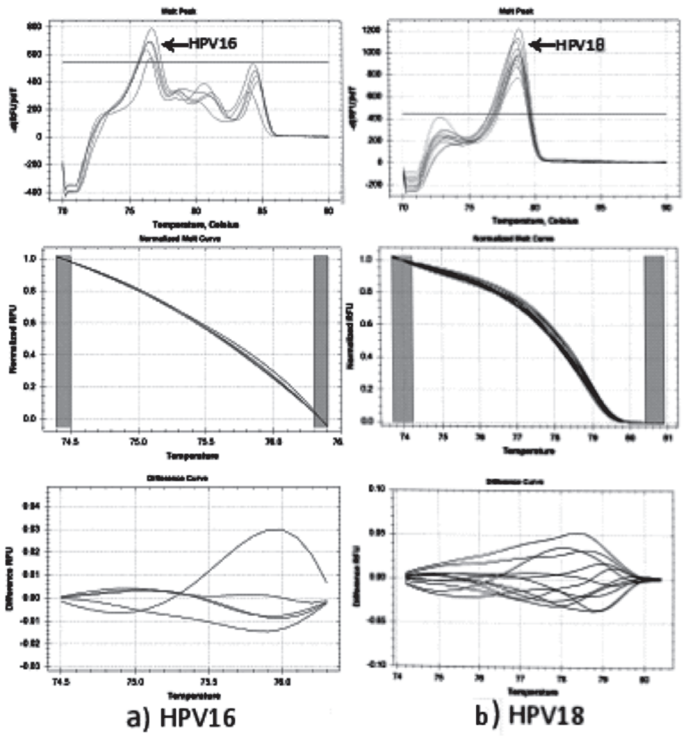

Figure 1: Precision melt analysis of HPV 16 and 18 by Bio- Red a) The 4 HPV16 positive amplicons and b) and 11 HPV18 positive amplicons, respectively. The negative derivative, the normalized melt curve and the difference curve plots were demonstrated in order in the upper, middle and lower rows. The melt curve shape sensitivity for cluster detection was set at 50% and the melting temperature difference threshold was 0.15°C for cluster detection. The pre-melt temperature for the HPV16 was 74.4°C to 74.5°C and the HPV18 were 73.7°C to 74.2°C whereas the post-melt region was 76.3°C to 76.4°C and 80.4°C to 80.9°C, successively.

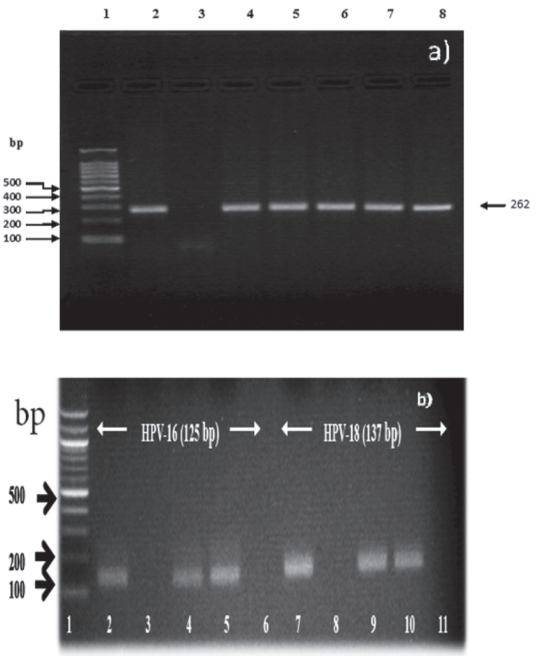

Figure 2. PCR products amplified by Agarose gel electrophoresis the β-globin primers and HPV-16 and -18 from nested PCR by

Table 1. Primers used in this study

Primers	Target region	Annealing temperature	Sequence of DNA	Amplicon size	Reference
KM 29	β-globin region	56.3°C	GGTTGGCCAATCTACTCCCAGG	262 bp	8
KM138			TGGTCTCCTTAAACCTGTCTTG		
HPV 16 F	E6 HPV region	57°C	GAGATGGGAATCCATATGCTG	125 bp	
HPV 16 R			CAACGGTTTGTTGTATTGCTG		
HPV 18 F	E7 HPV region	59.6°C	ATGTCACGAGCAATTAAGC	137 bp	9
HPV 18 R			TTCTGGCTTCACACTTACAAC		

Table 2. HPV 16 and 18 and association with investigated clinical parameter by the Pearson χ^2 test

Variables	Group	Total numbers	Numbers of HPV	P. Value
Age	16–19	6	0	0.227
	20–29	53	2	
	30–39	61	1	
	40–49	41	8	
	50–59	28	4	
	60–69	3	1	
Sex	Male	120	13	0.279
	Female	72	3	
Smoking	Non-smoker	102	3	0.001
	Former smoker	53	6	
	Current moker	37	7	
Betel quid chewing	Non chewer	151	9	0.266
	Former chewer	23	2	
	Current chewer	18	5	

the 125-bp and 137-bp PCR products were present in all of the HPV16 and HPV18 positive samples (Figure 2b).

The Pearson χ^2 test, results indicated that in this study the smoking subjects harbored more HPV than non-smokers ($p=0.001$); however, there was no association noted with other variables and the presence of HPV (Table 2).

a) Agarose gel electrophoresis of PCR products amplified by β-globin primers (KM 29 and KM 138) amplified product was showing 262 bp. Lane 1: Molecular marker 100 bp,Lane 2 : Hela cell positive control, Lane 3: Negative control, Lane 4-8 : DNA positive from β-globin b) Agrose gel Electrophoresis of HPV-16 and -18 PCR Products from nested PCR Analysis. Lane 1 represents 100-bp DNA ladder. Lanes 2-6 represent the detection of HPV- 16 using nested PCR; lane 2, positive control using SiHa DNA; lane 3, negative control using sterile distilled water; lanes 4 and 5, positive samples; lane 6, negative sample. Lanes 7-11 represent the detection of HPV-18 using nested PCR; lane 7, positive control using HeLa DNA; lane 8, negative control using sterile distilled water; lanes 9 and 10, positive samples and lane 11, negative sample.

DISCUSSION

Oral cavity is the gateway of entering the microorganisms like bacteria's, viruses, fungus and one of the most important viruses like HPV, which can be responsible for many diseases not only in oral cavity but other part of body too. In normal individuals, HPV presence is low but in faviourable conditins, like compromised immune system, theses number increase exponentially. High viral loads are believed to participate in the

pathogenesis of several cancers, including OSCC. So far, there have been only a few studies about the HPV 16 and 18 loads in healthy oral mucosa. This study was conducted to investigate the presence of HPV 16 and 18, and its relation to high risk factors among Dental Patients who attended the OPD for normal Oral Cavity checkup in Dental Section SPH Quetta.

HPV exists differently among various human tissues such as between in buccal swab and in peripheral lymphocytes[11]. Even in the similar origin, there are several reports indicating the presence of HPV in normal oral cavity with healthy mucosa however, ratio is different considerably. For instance, one study conducted by Sosorbaram *et al*. reported the number of HPV of 25% in normal oral cavity of 192 Mongolians, aged between 1 to 20 years old[12]. This is the agreement to the findings of this study, which revealed the prevalence of (8.3%) HPV 16 and 18 in normal epithelial cells, among these, 4 samples HPV 16 and 11 samples were HPV 18, and one sample was positive for both strains HPV 16 and HPV 18 strain. However, Ha *et al.* revealed the low incidence of HPV16 in premalignant and malignant lesions in the oral cavity using the Real-Time PCR. HPV was detected 1 out of 102 premalignant lesions, 1 out of 34 invasive oral cavity carcinoma and 14 out of 18 known HPV positive tumors[13]. This may partly due to differences in types of population, sample size, sampling procedure as well as detection assays. These factors are of significance when comparison among these data is performed.

In this study the statistical Pearson χ^2 test was used to analyze the association between the presence of HPV and clinical parameters. The number of positive cases of HPV 16 AND 18 in this study was higher in male as compare to female. This is the similarity with the findings of previous research work done in different part of the world as Male are more defenseless to HPV transmission risk factors as compare to Female[14]. It is still debatable that whether the presence of HPV is based on age or not because overall HPV infection percentage is noted high in young individuals as compare to old age with the two peak ages being less than 25 years as well as above 45 years old[15,16]. However, there is one study indicating a decrease of HPV in those older than 50 years[17]. The findings of this study disclose that the incidence of HPV increased with age from 20-59 years old. This may result from the longer exposure period to HPV as well as its risk factors. One of these majorities of respondents HPV positive accustomed to smoking. Since smoking interferes the immune system, which defects cytokine production and endorses viral survival. This is of importance because variations in tobacco and alcohol use play a contributing factors with association of HPV have a strong influence on the high prevalence rate of Cancers of the Oral cavity and posterior part of the throat around the world[18]. However betel quid chewers did not associated with high prevalence rate in this study.

The presence of HPV in this study showed low level with normal oral cavity but previous studies suggests that Oral cavity may be a favorable for such kind of microorganism especially for HPV without any clinical presentation. However there is no strong linkage about the presence of HPV and their increased numbers of viral loads are associated in the viral progression of the HPV related lesions and development of Oral Cancers[19]. And it is the need of time to find out the important potential factors to reduce the viral transformation and viral load for good prevention and HPV associated lesions[20,21].

In summary, the present study employed the sensitive Real-Time PCR for HPV detection in normal oral mucosa without any red and white lesions, the prevalence of HPV 16 and 18 in normal oral epithelial cells among the investigated patients were 8.3%, more HPV18 than HPV16 positive samples. The numbers of HPV 16 and 18 was low suggesting subclinical or latent HPV infection in normal oral cavity. One interesting clinical parameter smoking use association with viral load. Thus the health care workers, especially dentists, should aware of the low HPV loads in healthy subjects and educate their patients regarding the link of HPV related lesions in oral cavity.

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REFERENCES

1. Cason J, Mant CA. High-risk mucosal human papillomavirus infection during infancy & childhood. J Clin Virol. 2005;32:S52-8.
2. Kojima A, Maeda H, Kurahashi N, Sakagami G, Kubo K, Yoshimoto H, Kameyama Y. Human papillomaviruses in the normal oral cavity of children in Japan. Oral Oncol. 2003;39:821-8.
3. D'Souza G, Kreimer AR, Viscidi R, Pawlita M, Fakhry C, Koch WM, Westra WH, Gillison ML. Case-control study of human papillomavirus and oropharyngeal cancer. N Engl J Med. 2007;356:1944-56.
4. Raza SA, Franceschi S, Pallardy S, Malik FR, Avan BI, Zafar A, Ali SH, Pervez S, Serajuddaula S, Snijders PJ, van Kemenade FJ, Meijer CJ, Shershah S, Clifford GM. Human papillomavirus infection in women with and without cervical cancer in Karachi, Pakistan. Br J Cancer. 2010;102:1657-60.
5. Petersen PE. The World Oral Health Report 2003: continuous improvement of oral health in the 21st century--the approach of the WHO Global Oral Health Programme. Community Dent Oral Epidemiol. 2003;31:s3-23.
6. Nagpal JK, Patnaik S, Das BR. Prevalence of high-risk human papilloma virus types and its association

with P53 codon 72 polymorphism in tobacco addicted oral squamous cell carcinoma (OSCC) patients of Eastern India. Int J Cancer. 2002;97:649-53.

7. Khovidhunkit SO, Buajeeb W, Sanguansin S, Poomsawat S, Weerapradist W. Detection of human papillomavirus in oral squamous cell carcinoma, leukoplakia and lichen planus in Thai patients. Asian Pac J Cancer Prev. 2008;9:771-5.

8. Gravitt PE, Peyton C, Wheeler C, Apple R, Higuchi R, Shah KV. Reproducibility of HPV 16 and HPV 18 viral load quantitation using TaqMan real-time PCR assays. J Virol Methods. 2003;112:23-33.

9. Boy S, Van Rensburg EJ, Engelbrecht S, Dreyer L, van Heerden M, van Heerden W. HPV detection in primary intra-oral squamous cell carcinomas--commensal, aetiological agent or contamination? J Oral Pathol Med. 2006;35:86-90.

10. Ha PK, Califano JA. The role of human papillomavirus in oral carcinogenesis. Crit Rev Oral Biol Med. 2004;15:188-96.

11. Sosorbaram K, Sugita Y, Suzumura Y, Sato E, Maeda H and Kameyama Y. Human papillomavirus infection in the normal oral cavity of young people in Mongolia. Oral Med Pathol 2006; 11: 105- 112

12. Esquenazi D, Bussoloti Filho I, Carvalho Mda G, Barros FS. The frequency of human papillomavirus findings in normal oral mucosa of healthy people by PCR. Braz J Otorhinolaryngol. 2010;76:78-84.

13. Ha PK, Pai SI, Westra WH, Gillison ML, Tong BC, Sidransky D, Califano JA. Real-time quantitative PCR demonstrates low prevalence of human papillomavirus type 16 in premalignant and malignant lesions of the oral cavity. Clin Cancer Res. 2002;8:1203-9.

14. Reiter PL, Brewer NT, Smith JS. Human papillomavirus knowledge and vaccine acceptability among a national sample of heterosexual men. Sex Transm Infect. 2010;86:241-6.

15. Kulasingam SL, Hughes JP, Kiviat NB, Mao C, Weiss NS, Kuypers JM, Koutsky LA. Evaluation of human papillomavirus testing in primary screening for cervical abnormalities: comparison of sensitivity, specificity, and frequency of referral. JAMA. 2002;288:1749-57.

16. Herrero R, Hildesheim A, Bratti C, Sherman ME, Hutchinson M, Morales J, Balmaceda I, Greenberg MD, Alfaro M, Burk RD, Wacholder S, Plummer M, Schiffman M. Population-based study of human papillomavirus infection and cervical neoplasia in rural Costa Rica. J Natl Cancer Inst. 2000;92:464-74.

17. Cotton SC, Sharp L, Seth R, Masson LF, Little J, Cruickshank ME, Neal K, Waugh N; TOMBOLA Group. Lifestyle and socio-demographic factors associated with high-risk HPV infection in UK women. Br J Cancer. 2007;97:133-9.

18. Spence AR, FE, Ferenczy A. The role of human papillomavirus in cancer. Am J Cancer. 2005;4:49–64.

19. Ha PK, Califano JA. The role of human papillomavirus in oral carcinogenesis. Crit Rev Oral Biol Med. 2004;15:188-96.

20. Esquenazi D, Bussoloti Filho I, Carvalho Mda G, Barros FS. The frequency of human papillomavirus findings in normal oral mucosa of healthy people by PCR. Braz J Otorhinolaryngol. 2010;76:78-84.

21. Clavel C, Masure M, Putaud I, Thomas K, Bory JP, Gabriel R, Quereux C, Birembaut P. Hybrid capture II, a new sensitive test for human papillomavirus detection. Comparison with hybrid capture I and PCR results in cervical lesions. J Clin Pathol. 1998;51:737-40.