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Detecting Human Papillomavirus 6 & 11 in a Set of Pakistani Population

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Authors' contributions

Authors FP and SB designed the study. Author FP collected the samples, author FP did the bench work, wrote the protocol, and wrote the first draft of the manuscript. Authors SB and NM facilitated in literature search and finalization of the manuscript. Author FA assisted in analyses of the data. All authors read and approved the final manuscript.

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ABSTRACT

Introduction: Human papillomavirus 6 &11, two clinically important low risk-HPV genotypes are known for genital warts, laryngeal papillomas and low-risk squamous intraepithelial lesions. Recent research has documented HPV 6 &11, cause of malignancies in oral cavity, pharynx, larynx etc. the frequency of which is also increasing in Pakistan. Therefore, it was hypothesized that HPV 6 & 11 might also be associated with oral malignancies in Pakistan.
Methodology: For this case control study a total of 266 subjects (133 cases and 133 controls), >18yrs, smokers and chewers were recruited. Diagnosed cases of Squamous Cell Carcinoma of the oral cavity, larynx and pharynx were included. Precancerous oral lesions were identified on the basis of Axell & Poulson criteria. According to their classification, subjects falling in grade 1 or above were considered as oral lesion cases. The subjects without any signs similar to grade 1 and without any lesions were controls. Detailed questionnaire was filled followed by oral sample

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collection. After DNA extraction conventional PCR for HPV was performed. Positive samples were further analyzed for HPV 6 &11 on Real time PCR.

Results: The 266 subjects (mean age cases-42.07±13.7; controls- 33.61±11.68 years) included 132 males [93 (69.9%) cases, 39 (29.3%) controls] and 134 females [40 (30.1%) cases, 94 (70.7%) controls]. Further division into six ethnic groups showed Urdu speaking as major group (40.6% cases and 38.3% controls). Main habit reported was Gutka (67; 25.18%). The most common (68; 95.77%) cancer was Squamous Cell Carcinoma with buccal mucosa (49; 69.01%) as the most common site. HPV was found in 21 (7.9%) cases only. Strong association of HPV with cases (*P*= 0.006) was found at confidence interval of 3.06-851.8 and odds ratio of 51.02. However none was positive for HPV 6&11. The HPV association was significant with betel quid addiction (*P*<0.0001). The most common (68; 95.77%) cancer observed was Squamous Cell Carcinoma of the oral cavity with buccal mucosa (49; 69.01%) as the most common site.

Conclusion: Since, HPV 6/11 was not detected we can conclude that there is no association between HPV 6/11 and tobacco use leading to oral lesions/cancers. Thus there is no definitive causative role of HPV 6&11 in oral carcinogenesis in our ethnically diversified population where use of chewable tobacco is very common among the masses.

Keywords: Human papillomavirus; HPV6; HPV11; chewable tobacco; carcinoma; squamous cell.

1. INTRODUCTION

Human papillomavirus (HPV), a circular non-enveloped, double-stranded DNA virus has about 195 genotypes which have been sequenced and recognized [1]. HPV genotypes 6 &11 are classified in the alpha HPV genus. HPV 6 & 11, well known for causing genital warts, laryngeal papillomas and low-risk squamous intraepithelial lesions were earlier considered as the two clinically most important low risk-HPV genotypes [2]. Later on association of HPV 6 & 11 was reported with various types of malignancies such as Squamous Cell Carcinoma of the Conjunctiva [3], lung carcinoma [4], laryngeal carcinoma [5], malignant progression in laryngeal papilloma [6], malignant transformation in recurrent respiratory papillomatosis [7], penile [8], urothelial carcinoma [9], vulva [10], cervical carcinomas [11] and high grade lesion and anal cancer [12].

Generally, HPV infection is transmitted through sexual or asexual behavior [13]. However, oral HPV infection can be transmitted by contact through oral-genital, mouth-to-mouth, autoinoculation or vertical transmission in infants from mother-to-child [14]. Smokers, tobacco chewers, male gender, many sexual partners and people with weak immune system are at a greater risk of acquiring this infection [15].

Continuous exposure of oral mucosa to tobacco and other ingredients added in these chewable formulations cause abrasion which leads to desquamation and penetration of HPV into basal layer of squamous epithelium where it replicates and integrates into human DNA. Its E6 and E7 protiens bind to DNA and inhibit p53 and pRb gene. Inhibition of these two important tumour suppressor genes results in development of certain malignancies such as squamous cell carcinoma of oral cavity, pharyngeal or laryngeal carcinomas [16]. The frequency of these carcinomas is increasing in Pakistan [17,18]. Oral carcinoma ranks 8th world-wide. However, 58% of the cases are concentrated in South and Southeast Asia. In Karachi, it ranks 2nd in all malignancies among both males and females, with the highest reported incidence in the world [19]. Since, Worldwide, HPV 6 and 11 are being mapped for number of reported case of malignancies, therefore, it was important to find out its association with oral lesion and cancer in Pakistan.

2. MATERIALS AND METHODS

This case control study was conducted from August 2014 to December 2015 at Ziauddin University Karachi, after approval by the Ethical Review Committee of Ziauddin University, Karachi (Reference no. 0140614FPBIO).Sample size was calculated using case control formula with odds ratio of 2 which was detected from a previous study [20] Intraoral examination was done for the presence or absence of oral lesions of all subjects with the help of trained dentists, using mirror and gauze. Patients≥ 18years of age, whether or not habitual tobacco users (smokers and chewers) were included. The lesions were identified on the basis of Axell et al. [21] and Greer & Poulson [22] criteria. According to their classification, oral lesions were divided into 3 grades. The subjects falling in grade 1 or

above were considered as oral lesion cases. Diagnosed cases of OSCC, laryngeal and pharyngeal carcinomas were also included in this case control study. The subjects without any signs similar to grade 1 and without any lesions were controls. Subjects addicted to substances other than tobacco in any form and patients not willing to give written inform consent were excluded. All samples were collected as 40 ml oral rinse with gentle brushing of the oral mucosa with the help of a brush at the other end of dental floss and stored at 4ºC till DNA extraction. Demographic data and details of chewing habits were noted by using a questionnaire. DNA was extracted through a protocol previously reported [23]. The DNA quantification was done by using Qubit® dsDNA BR Assay kit (Qubit® 2.0 Invitrogen life technologies USA).

2.1 Polymerase Chain Reaction

The PCR was performed on all samples with L1 gene specific consensus primers Gp5+/ Gp6+. Primers sequence for HPV- Gp5+ was 5'- TTTGTTACTGTGGTAGATACTAC-3', and HPV-Gp6+ was 5'- GAAAAATAAACTGTAAATCATATTC-3' (Gene Link NY USA). DNA amplification was done in BIOFLUX, a conventional thermo cycler. The PCR amplification was carried out in a volume of 25 µl containing 12.5 µl of master mix (Promega). The cycling was performed for 5 min at 94ºC; followed by 35 cycles of PCR consisting of denaturation for 30 sec at 94ºC, annealing for 30 sec at 55°C, and extension for 1 min at 72º, and a final extension for 5 min at 72ºC. The amplified product of150 base pair for HPV was analyzed on 2% Agarose gel by electrophoresis [23]. DNA was amplified for HPV 6 & 11 with specific primers (6A 5'-TAGGGGACGGTCCTCTATTC-3, 6B 5'-GCAACAGCCTCTGAGTCACA-3,11A 5'-GAATACATGCGCCATGTGGA-3'and 11B 5'-AGCAGACGTCCGTCCTCGAT-3') through a protocol previously reported [24].

2.2 Real Time Polymerase Chain Reaction for HPV 6 &11

Real time PCR was performed on HPV positive samples by using human papillomavirus (type 6 and 11) DNA fluorescence diagnostic kit (Fast HPV 6/11 by Sansure Biotech).

3. RESULTS

In total, 266 subjects including 132 males [93 (69.9%) cases, 39 (29.3%) control] and 134 females [40 (30.1%) cases, 94 (70.7%) controls] were included in the study. The mean age of the cases was 42.07±13.7 and control was 33.61±11.68 years. They were further divided into six ethnic groups and majority of the subjects, 40.6% cases and 38.3% controls were Urdu speaking Table 1.

Table 1. Demographic details of the studied population

Variable		Case		Control	
		N	%	N	%
Gender	Male	93	69.9	39	29.3
	Female	40	30.1	94	70.7
Ethnicity	Balochi speaking	10	7.5	1	.8
	Sindhi speaking	8	6.0	4	3.0
	Punjabi speaking	7	5.3	11	8.3
	Pushto speaking	36	27.1	52	39.1
	Urdu speaking	54	40.6	51	38.3
	Others	18	13.5	13	9.8
Age		Mean	SD	Mean	SD
		42.07	13.7	33.61	11.68

Our study showed that HPV is strongly associated with positive cases with a (*P*=<0.006) at confidence interval of 3.06-851.8 and odds ratio of 51.02 (Fig. 1).

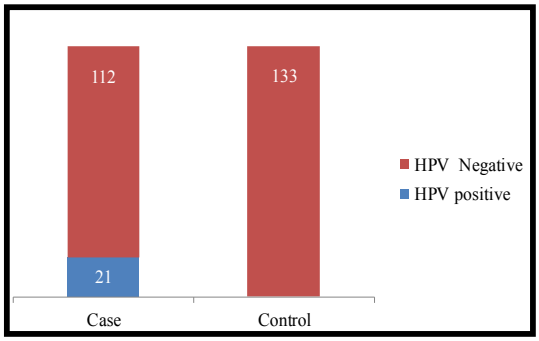

Fig. 1. Frequency of HPV incases and controls

In the cases 62 were pre-cancerous while 71 were cancers. These 71 cancers include OSCC (19 HPV positive, 49 HPV negative), Pharyngeal (2 and both HPV negative) and one HPV negative laryngeal carcinoma.

Strong association was found between HPV and ethnicity (*p* .02). The frequency of HPV among different ethnicities is shown in Fig. 2, with the

highest number of HPV in Urdu speaking 12(57.1%).

The presence of HPV was confirmed by conventional PCR using HPV general primers (Fig. 3).

HPV was found in 21 (7.9%) cases only, however, none of these were positive for HPV 6&11. PCR results revealed 21 HPV positive cases in our sample. (112 HPV negative cases with all negative controls i.e. 133).

In the 21 HPV positive cases further genotyping revealed absence of HPV 6/11. For validation of the results we also performed real time PCR. Results of RT- PCR confirmed results of conventional PCR, i.e. absence of HPV 6/11 in our samples (Fig. 4).

All the cases were addicted to tobacco in some form. Majority reported Gutka (67; 25.18%) as the main habit Table 2.

The most significant association between tobacco consumption and HPV was found to be with betel quid (*P*=0.0001) Table 2.

The most common (68; 95.77%) cancer observed was squamous cell carcinoma of the oral cavity with buccal mucosa (49; 69.01%) as the most common site of OSCC Table 3.

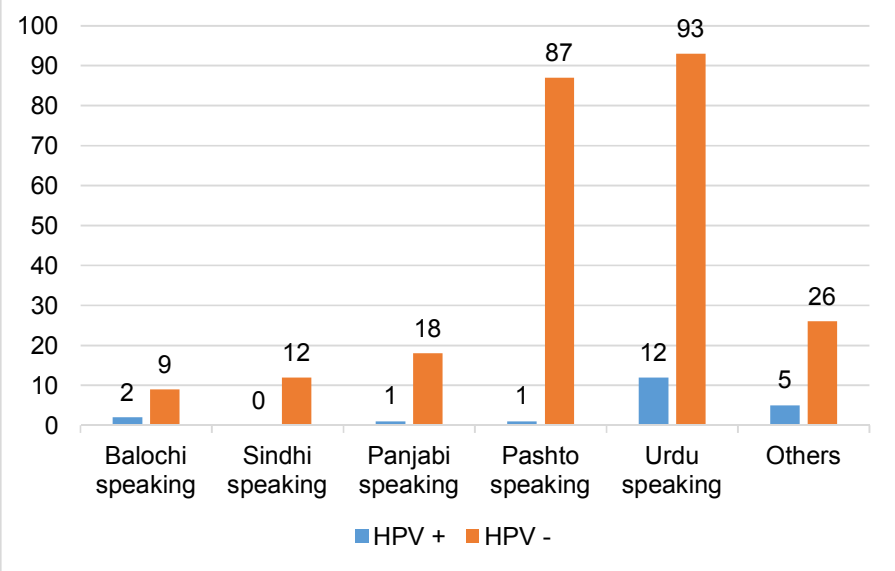

Fig. 2. Frequency of HPV in different ethnicities (*P*=.023)

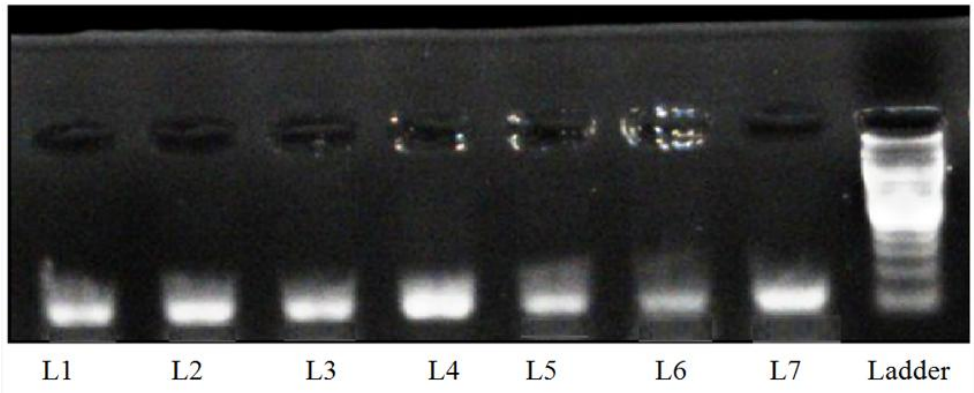

Fig. 3. Gel electrophoresis of HPV general primers PCR
Ladder:100 bp DNA ladder, Lanes (1-7): Represents HPV

Fig. 4. Quantitative real-time PCR to determine HPV 6 &11 viral load in DNA isolated from oral lesion and cancer patients

DNA of HPV positive oral lesion and cancer samples were amplified (as described in Methods). A: DNA amplification threshold cycle analysis; B: standard curve analysis as the slope for determining the efficiency of reaction. C: Multicomponent plot and D: Raw data plot

Table 2. Types of tobacco habits and association with HPV

Variable		HPV		Odds ratio	CI	*P* value
		Positive	Negative			
Naswar	Yes	2	57	0.35	0.07-1.53	0.16
	No	19	188			
Gutka	Yes	9	58	2.4	0.97-6.0	0.051
	No	12	187			
Areca Nut	Yes	3	61	0.5	0.14-1.8	0.28
	No	18	184			
Betel Quid	Yes	13	47	6.8	2.7-17.4	0.0001
	No	8	197			
Cigarette	Yes	8	42	2.97	1.16-7.6	0.02
	No	13	203			

4. DISCUSSION

Despite a high frequency up to 31.9% of HPV in general chewable tobacco population of Pakistan [23], HPV 6/11 was not detected in this study done in the same setting on tobacco chewers suffering from oral cancer. The HPV study previously conducted on different ethnicities reported a range from 8.5% to 31.9% frequency of HPV in general population [23]. Further genotyping of HPV in the previous studies revealed presence of HPV 16 and 18 in addition to others which could not be typed [25]. The present study is believed to be the first of its kind in Pakistan that investigated HPV 6&11. In this study, HPV was found in 7.9% of samples, however, when further genotyping of HPV 6 and 11, in pharyngeal or laryngeal squamous cell carcinoma patients was done, none of the samples revealed their presence whereas, studies around the world have shown that HPV has been implicated to play a role in the etiology of oral, pharyngeal and laryngeal squamous cell carcinoma [18,26].

Comparing various studies on HPV, the research on HPV 6/11 has been found highly variable and controversial. Maden et al. reported that men with an oral HPV-6 infection had 2.9 times the risk for oral cancer of non-infected men (95% CI 1.1–7.3) [27]. A study conducted in Wuhan, China on OSCCs detected HPV6 as the only low-risk type, nonetheless, HPV16 and HPV18 were identified as the main types [28]. A study conducted in early nineties reported about the prevalence and type-specific distribution of HPV in neoplastic and non-neoplastic cervical tissues by in situ hybridization. HPVs [16,18] were found in 17% of the cases, whereas double infection with HPV 16 and 6/11 was detected in 6 (8%) [29].

Table 3. Association of HPV with Type and location of squamous cell carcinomas and precancerous lesion

Squamous cell carcinomas	HPV positive N=19		HPV negative N=52
Oral cavity (n=68)	Tongue	6	
	Left buccal mucosa	4	49
	Right buccal mucosa	9	
	Pre-cancerous	2	
Pharynx (n=2)	0		2
Larynx (n=1)	0		1

The burden of these carcinomas is on the rise not only in Pakistan [30] but also worldwide [31]. It is reported that tobacco and HPV infection supplement each other in the growth of these carcinomas [32].

In our study all the cases had a history of tobacco chewing and smoking. Majority of patients reported the use of Gutka (67; 25.18%) as the main habit. Similar pattern of tobacco usage was observed by other study [18]. Squamous cell carcinoma of the oral cavity was observed as the most common type of cancer (68; 95.77%) with male predominance in our study, which is in accordance with other studies [33]. This is probably the chewing of tobacco products is more common in males [34]. This supports the fact that males are more prone to develop OSCC compared to females due to exposure to the risk factors like tobacco and HPV. The ethnic group mostly affected in our study was Urdu speaking; this is in accordance with the findings of Aamir S [35]. Chewing betel quid or paan is an inherited tradition of central and South Indian culture for hundreds of years and this habit migrated along with this group to this part of sub-continent and adopted by other ethnicities.

The most commonly involved site of OSCC in this study was buccal mucosa (49; 69.01%); finding which was also reported by Mirza S and Jayasooriya [36,37]. The possible reason, for buccal mucosa as the commonest site, was probably due to prolonged exposure of buccal mucosa with chewable tobacco as it is kept in buccal pouch. Some other studies reported that the most common site of OSCC is tongue and lip [38]. The discrepancy of these results could be because of difference in risk factors. OSCC of floor of the mouth and tongue may be associated with excessive alcohol consumption, cigarette-smoking, HPV infection and sexual behavior.

The overall prevalence of oral HPV infection in our study was 7.9%. These findings are quite close to studies reported by Zafar and Emmett [33,39]. There are wide differences in the findings of different studies. Gichki et al. reported HPV prevalence 24.5% in a group of Pakistani subject having normal oral cavity visiting the dental department. They used oral tissue scraping for DNA detection by Real-time PCR method [20]. Marur et al. Reported 17.7% cases of OSCC positive for HPV, they performed Dot Blot, in-situ hybridization and PCR method [40]. Possible reasons of this discrepancy is variation in samples collection techniques, preparation method, and different type of molecular assay.

The most significant association between tobacco consumption and HPV was found with Betel Quid (<0.0001), the areca nut component of these products has tendency of eroding the oral mucosa which further contributes to development of rough mucosa [41]. Association with Areca Nut (0.28) and Naswar (0.16) were not significant probably because of Naswar is not chewed and is simply kept under the lower lip or tongue or inside the cheek and sucked [18,42].

Since testing of other genotypes was not done, therefore, a definitive genotype cannot be held accountable for the OSCC in our patients, but previous literature has shown the presence of 16 & 18 in Pakistani population [43], which could be the underlying cause.

5. CONCLUSION

In spite of scanning all types of tobacco users, chewers and smokers, suffering from oral lesions or OSCC, HPV 6/11 was not detected. Hence we can conclude that there is no association between HPV 6/11 and tobacco use leading to oral lesions/cancers. Thus there is no definitive causative role of HPV 6&11 in oral carcinogenesis in our ethnically diversified population where use of chewable tobacco is very common among the masses.

6. LIMITATIONS

Sample size was small and other genotypes could not be checked.

7. STRENGTH OF THE STUDY

Conventional as well as real time PCR both were done for the confirmation of absence of both the strains included in the study.

8. FURTHER DIRECTIONS

A cross sectional study with larger sample size of oral lesion /cancer should be conducted.

CONSENT

A written informed consent was obtained from each subject.

ETHICAL APPROVAL

Ethical approval was taken by the Ethical Review Committee of Ziauddin University, Karachi (Reference no. 0140614FPBIO).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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