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Salivary Detection of Viral DNA Loads of HPV 16 and 18 in Oral Potentially Malignant Disorders and Oral Squamous Cell Carcinoma

[1]Dr. Syed Afroz Ahmed, MDS, Ph. D. Scholar, Pacific Academy of Higher Education & Research University, Udaipur

[2]Dr. Prashant Nahar, MDS, Professor, Department of Oral Medicine and Radiology, Pacific Academy of Higher Education & Research University, Udaipur

[3]Dr. Vaddalapu Hari Priya, MDS, Oral Pathologist

[4]Dr. Alimullah Fawaz, MBBS, MD, Professor, Department of Microbiology, Sri Sai College of Dental Surgery, Vikarabad

[5]Dr. Tammali Vasavi, (MDS), Post Graduate Student, Department of Oral and Maxillofacial Pathology, Sri Sai College of Dental Surgery, Vikarabad

[6]Dr. Asra Tahemina, (MDS), Post Graduate Student, Department of Oral and Maxillofacial Pathology, Sri Sai College of Dental Surgery, Vikarabad

Corresponding Author: Dr. Syed Afroz Ahmed, MDS, Ph. D. Scholar, Pacific Academy of Higher Education & Research University, Udaipur

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Introduction

Oral cancer or Oral Squamous Cell Carcinoma (OSCC) is the 6th commonest cancer in the world1especially in South and Southeast Asia, with India reporting high incidence rates. Oral cancer represents the leading cause of death[3]. Most cases of oral cancer are associated with habits (tobacco/areca nut) and are generally preceded by asymptomatic clinical lesions collectively known as Oral Potentially Malignant Disorders (OPMD). OPMD's include oral leukoplakia, erythroplakia, reverse smoker's palate, oral lichen planus, oral submucous fibrosis (OSMF) and actinic keratosis[4]. A variety of viruses are also responsible for causing OSCC, most common being Human Papilloma Virus (HPV). High-risk types of HPV include HPV-16, 18, 31, 33, 45, 51, 52, 56, 58, 59 & low-risk types include HPV-6, 11, 42, 43, 44. [8] The paper highlights a strong correlation between high-risk HPV types and the development of OSCC, noting that

HPV-16 and HPV-18 are frequently detected in both malignant oral lesions and OPMDs.

High risk subtypes have been identified in the majority of malignant lesions whereas, low-risk types are often associated with benign lesions[9]. Persistent HPV infection in the oral mucosa might increase the risk of developing oral cancer in a population younger than that typically affected with HPV independent oral cancers [5] . The reported rates of HPV while Deoxyribo Nuecliec Acid (DNA) detection in OPMD's and OSCC range from 0% to 100% [4]. This extreme variation indicates the difference in ethnicity, geographic locations, type of samples collected, patient group selected to variations in methods used for HPV detection. Hence, part of HPV role in OPMD's and OSCC remains obscure [(1,10)]

While traditional histopathological methods are the standard for diagnosis, the authors in this study recommend molecular techniques such as real-time polymerase chain reaction (RT-PCR) due to their higher sensitivity and specificity for detecting HPV DNA. Saliva is presented as a non-invasive sample source for HPV detection, making it suitable for widespread screening. The study concludes that HPV-16 and HPV-18 significantly contribute to the pathogenesis of oral cancer. Early detection of these viruses in OPMDs could be crucial for preventing progression to OSCC. The findings advocate for the integration of molecular diagnostic techniques in clinical practice to enhance early diagnosis and intervention strategies, ultimately improving patient outcomes in populations at risk for oral cancer.

Material and Methods

The present case-control study was done on 45 samples which included 15 healthy individuals as controls along with that 15 histopathologically diagnosed cases of OPMD's & 15 cases of OSCC were taken as case groups. The samples were retrieved from Sri Sai College of Dental Surgery, Vikarabad in the Department of Oral and Maxillofacial Pathology. Ethical clearance for conducting the study was obtained from the Institutional Ethical Clearance Committee. An informed consent was obtained from study subjects selected to be a part of the study. The relevant demographic data including name, age, sex and purpose of sample collection was recorded for each individual.

Samples were selected on the basis of inclusion and exclusion criteria as mentioned below:

Inclusion criteria: Saliva samples obtained from OPMD group, OSCC group & healthy individuals.

Exclusion criteria: Patients with saliva containing blood & food debris, medical & surgical history; drug addictions.

The materials used in the study were as follows:

Eppendorf tubes HiPurA® Viral DNA Purification Kit was used which consisted of the following:

1. Resuspension Solution
2. Lysis Solution
3. Ethanol
4. Prewash Solution Concentrate
5. Wash Solution Concentrate
6. Elution Solution (RNase- Free w
7. Proteinase K
8. Carrier RNA
9. HiElute Miniprep Spin Column (Capped)
10. Collection Tubes (Uncapped)
11. Polypropylene

Collection Tubes Hi-PCR® Human Papilloma Virus (HPV) Genotyping (16,18 & 45 Multiplex) Probe PCR Kit

1. Hi-Quanti 2x Realtime PCR Master MIX
2. II. HPV Multiplex Primer-Probe Mix
3. Internal Control T DNA

4. HPV Multiplex Positive Control
5. Molecular Biology Grade Water for PCR.

Methodology

Saliva sample collection: - Subjects were asked to rinse the mouth thoroughly using distilled water to remove any food debris. Later, subjects were trained to collect the saliva in the floor of the mouth for whole saliva collection. Then they were allowed to fill the sample collection container by spitting method

Sample transport and storage: Samples collected were transported to the lab by using VTM and stored at -20°C.

DNA isolation: Procedure:

1. Add 25 µl of the reconstituted Proteinase K solution (20 mg/ml) into 2.0 ml capped collection tube containing 200 µl of plasma, serum or body fluid. Vortex for 10-15 seconds to ensure thorough mixing.
2. Lysis reaction - Add 200 µl of the Lysis Solution (C1)- Carrier RNA to the sample, vortex thoroughly for 15 seconds to obtain a homogenous mixture. Incubate at 56°C for 15 minutes.
3. Centrifuge the samples for 10 seconds to remove droplets formed inside the cap of the collection tubes.
4. Prepare for Binding - Add 250 µl of ethanol (96-100%) to the lysate obtained from the above step for preparation of lysate for binding to the spin column. Mix thoroughly by gentle pipetting. Incubate the lysate for 5 minutes at room temperature (15-25°C).
5. Centrifuge the samples for 10 seconds to remove droplets formed inside the cap of collection tubes.
6. Load lysate in HiElute Miniprep Spin Column (Capped) - Transfer the lysate obtained from step 5 into the spin column provided. Centrifuge at 8,000 rpm for 1 minute. Discard the flow-through liquid and place the column in same 2.0 ml collection tube.
7. Prewash - Add 500 µl of diluted Prewash Solution to the column and centrifuge at 8,000 rpm for 1 minute. Discard the flow-through liquid and re-use the same collection tube with the column.
8. Wash - Add 500 µl of diluted Wash Solution to the column and centrifuge at 8,000 rpm for 1 minute. Discard the flow-through liquid and re-use the same collection tube with the column.
9. Second wash - Add 500 µl of Ethanol (96-100%) to the column and centrifuge at 8,000 rpm for 1 minute. Discard the flow-through liquid and re-use the same collection tube with the column.
10. Place the column in a new collection tube and spin the empty column for another minute at 14,000 rpm for 3 minutes to dry the membrane.
11. Place the column in new collection tube and incubate at 56°C for 3 mins to dry the column.
12. DNA Elution - Pipette 20-150 µl of the Elution Buffer (RNase free water) directly onto the column without spilling to the sides. Incubate for 1 minute at room temperature (15- 25°C). Centrifuge at 13,000 rpm for 1 minute to elute the DNA. XIII. Transfer the eluate to a fresh capped 2ml collection tube for longer DNA storage 3

Reagents in viral DNA purification kit:

Reagents Resuspension Solution (1X PBS)
Lysis Solution (C1)
Prewash Solution Concentrate (PW)
Wash Solution Concentrate (WS)
Elution Solution (RNase- Free Water)
Proteinase K
Carrier RNA
HiElute Miniprep Spin Column (Capped)
Collection Tubes (Uncapped),
Polypropylene (2.0 ml) Collection Tubes,
Polypropylene (2.0 ml)

Master mix preparation:

Master mix was prepared as per the protocol given in the Hi-PCR® HPV Genotyping (16,18 & 45 Multiplex) Probe PCR kit.

Protocol for PCR Master Mix Preparation:

1. In the "Master mix Preparation" area, thaw all components from the kit on ice, mix by inverting the tubes and centrifuge the reagents for several seconds. Keep on ice for later use.
2. Based on the number of specimens to be tested (N), including the positive template control (PTC) and negative control (NC), calculate the volume of the components to be added as N* volume of 1X.
3. Use 1.5 mL Nuclease free centrifuge tube for the preparation of the reaction system. After all the reagents are added, mix them thoroughly and centrifuge for several seconds.
4. Load 19µL of master mix into the 0.1/0.2 mL PCR reaction tube/plate/strips, compatible to the instrument to be used; add 20 µL of master mixture with 5 µL Nuclease free water to the negative control.
5. In the "Nucleic acid handling" area, add 1 µL internal control target (T) DNA in each reaction. Further, add HPV Multiplex Positive Control and extracted test DNA into the plate/strip
6. Tightly cap the tubes/strips or seal the plate using an optically clear adhesive film.
7. Briefly, spin the strips/tubes to settle the reagent to the bottom of the tube.
8. Place the plate/strips/tubes in RT-PCR machine and set the PCR program.

Table 1: Components in PCR Primer

Components	Volume (µL) to be added for 1X (for a 25 µL reaction)
Hi-Quanti 2X Realtime PCR Master Mix	12.5
HPV Multiplex Primer-Probe Mix	3.0
Internal control Primer-Probe Mix	1.0
Internal Control T DNA	1
Molecular Biology Grade Water for PCR	2.5
Test – Extracted Sample DNA/ Positive Control – Provided in Kit/ Negative Control - Water	5
Total volume	25

PCR program

Prepared master mix was loaded into PCR wells by using 200 µl pipette.

The plate was sealed by an optically clear adhesive film.

The plate was placed in RT-PCR machine and PCR program was set.

Initial denaturation: 95°C for 10 minutes

Denaturation: 95°C for 15 seconds

Annealing: 55°C for 20 seconds (Plate Read)

Channel: FAM/Texas Red/JOE/Cy5

Hold: 4°C

Denaturation & Annealing channel No. of cycles: 40

Negative Control: A negative control is needed to ensure that the reagents,

equipment, and environment used in the assay is not contaminated. In this

reaction, nuclease free water is used as the template.

Positive control: This is a control reaction using a known template (target pathogen). A positive control is

usually used to ensure proper and intended functioning of all the reagents and is recommended to be used in every run to assess optimal performance.

Internal Control: This is a control sequence which is amplified in the same reaction tube along with the target sequence (target species) but detected with a different primer (i.e. Multiplex PCR). An internal control is often used to detect the failure of amplification in cases where the target sequence is not amplified.

Data Analysis:

a) HPV-16 was detected with FAM channel & HPV-18 was detected with Texas Red channel.

b) Positive and negative results were obtained by these channels.

Table 2: Cyclic Threshold (CT) Values

CT Value	Result
≤ 38	Detected (+)
> 38 or N/A	Not detected (-)

Obtained results were sent for statistical analysis. Statistical analysis was performed using computer with Statistical Package for Social Science software (SPSS VS 18).

Results and Observation

Table 3: HPV-16 Distribution in study group

Group	N	HPV 16 Positive	HPV 16 Negative	P Value
OPMDS	15	08	07	0.0
OSCC	15	10	05	0.0
CONTROL	15	00	00	0.0

Table 4: HPV-18 Distribution in study group

Group	N	HPV 18 Positive	HPV 18 Negative	P Value
OPMDS	15	03	12	0.11
OSCC	15	04	11	0.11
CONTROL	15	00	00	0.11

P value was statistically significant (P= 0.0) by Chi-square test in case of HPV 16 and statistically insignificant (P=0.11) in HPV 18.

Discussion

HPV shows a specific tropism for keratinocytes in epithelial tissues, integrating its DNA into host cells. HPV is divided into high-risk and low-risk types based on its role in cervical carcinogenesis. The viral oncoproteins trigger unregulated cell proliferation, leading to the immortality of keratinocytes, and result in excessive viral DNA production and chromosomal abnormalities due to the inhibition of tumor suppressor factors.

In the head and neck region, HPV is predominantly found in the oropharynx and Waldeyer ring, with the palatine tonsils and the base of the tongue being the most common sites for HPV DNA detection. Oropharyngeal HPV-positive tumors occur up to six times more frequently than other head and neck tumors.

Research on the viral loads of HPV-16 and HPV-18 in salivary samples is limited. This study aimed to detect salivary DNA loads of HPV-16 and HPV-18 in patients with OPMDs, OSCC and control groups, while also analyzing the potential role of these viruses in OSCC pathogenesis.

In the OPMD group, 53% were found to be positive for HPV-16, consistent with Neilsen et al. (1996)[19], who reported a 40.8% prevalence of HPV-16 in proliferative verrucous leukoplakia cases. Additionally, Fonseca-Silva et al. (2012)[2] identified HPV-16 in 69.2% of epithelial dysplasia cases, suggesting its involvement in dysplasia development.

Among OSCC cases, HPV-16 was detected in 67% of samples, aligning with Elango KJ et al. (2011)[22], who found a 48% prevalence in oral cancer patients, indicating a positive correlation between HPV-16 and

oral tongue cancers. However, a study by Tokuzen N et al. (2021) [35]reported a low prevalence (1%) of HPV-16 in the Japanese population, suggesting a lower likelihood of this virus contributing to OSCC in that demographic.

In our study, while examining both OPMDs and OSCC, 53% and 67% tested positive for HPV-16, respectively, contrasting with Ha PK et al. (2002),[39] who found HPV-16 DNA in only 0.98% of proliferative lesions and 2.9% of invasive OSCC, suggesting a minimal role in malignant progression.

Regarding HPV-18, positivity was found in 20% of OPMD cases, which aligns with Sand L et al. (2000)[40] who reported a 27% prevalence in oral lichen planus cases. In OSCC, HPV-18 positivity was 27%, contrasting with Giovannelli L et al. (2002),[20] who reported an 87.5% prevalence, linking HPV infection to a higher risk of malignant lesions.

The study revealed high prevalence rates of HPV-16 (53%) and lower rates of HPV-18 (20%) in OPMDs, consistent with Zendeli-Bedjeti L et al. (2017).[27] In OSCC, 67% were HPV-16 positive and 27% were HPV-18 positive, suggesting a potential role of high-risk HPV types in these conditions.

Notably, no co-infections of HPV-16 and HPV-18 were detected in OSCC samples, differing from studies that reported significant co-infection rates. This study also assessed the viral loads, finding that HPV-16 was more prevalent and significantly associated with OSCC (P=0.00), indicating its possible role in the disease's pathogenesis. These findings support earlier studies that identified HPV-16 as a risk factor in OSCC development.

Conclusion

The incidence of oral cancer is on the rise, with approximately 80% of cases in India preceded by OPMDs. Understanding the etiology of OPMDs and OSCC is crucial, particularly the distinct pathogenesis associated with high-risk HPV types 16 and 18, as opposed to traditional risk factors like tobacco use.

HPV infections are more prevalent among the elderly and immunocompromised individuals, such as those with HIV, and those with cervical cancer, as there are similarities between the genital and oral epithelium. Many women with cervical cancer may also harbor oral HPV infections. Early detection of high-risk HPV is essential for reducing cancer progression in OPMDs.

Detection methods include exfoliative cytology, oral rinse, saliva, and various tissue samples. Among these, PCR is the most sensitive, capable of identifying viral DNA in minimal samples. This study aimed to detect HPV-16 and HPV-18 in saliva from 15 OPMD and 15 OSCC patients, using a viral DNA purification kit and PCR techniques.

Results showed that HPV-16 was more prevalent than HPV-18 in the samples, indicating a potential role for both in the pathogenesis of OPMDs and OSCC. However, the study's limitations include a small sample size and potential technical errors. Furthermore, studies with larger sample size are needed to know the pathogenesis of high-risk HPV types in OPMD’s and OSCC. Awareness regarding HPV vaccine should be given to prevent the viral oncogenesis.

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