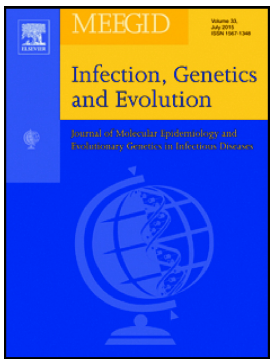

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Prevalence of Human Papillomavirus in the Oral Cavity of an Indigenous Community from Southwest México

Myriam Angélica de la Garza-Ramos[a], Victor Hugo Urrutia-Baca[b], Cynthia Sofia Urbina-Rios[b], Dabeiba Adriana García Robayo[c], Patricia Tamez-Guerra[b], Ricardo Gomez-Flores[b,*]

[a]Universidad Autónoma de Nuevo León, Facultad de Odontologia/Centro de Investigación y Desarrollo en Ciencias de la Salud (CIDICS), Calle Dr. Eduardo Aguirre Pequeño y Silao S/N, Colonia Mitras Centro. Monterrey, Nuevo Leon, México, CP. 64460.

[b]Universidad Autónoma de Nuevo León, Facultad de Ciencias Biológicas, Departamento de Microbiología e Inmunología, San Nicolás de los Garza, Nuevo León, México, C.P. 66455.

[3]Pontificia Universidad Javeriana, Centro de Investigaciones Odontológicas, Bogotá, Colombia.

Myriam Angélica de la Garza-Ramos, myriam.garzarm@uanl.edu.mx; Victor Hugo Urrutia-Baca, undervic1990@yahoo.com; Cynthia Sofia Urbina-Rios, cynthiasofiaurbinarios@gmail.com; Dabeiba Adriana García Robayo, garciad@javeriana.edu.co; Patricia Tamez-Guerra, patamez@hotmail.com; Ricardo Gomez-Flores, ricardo.gomezf@uanl.mx, rgomez60@hotmail.com.

Myriam Angélica de la Garza-Ramos and Victor Hugo Urrutia-Baca are the first authors

* Corresponding author: Tel: 5281(83) 29-40-00 ext. 6453. E-mail addresses: ricardo.gomezf@uanl.mx; rgomez60@hotmail.com.

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Abstract

Human papilloma virus (HPV) is a DNA virus associated with the development of cervical, penile, anal, vulvar, and oral cancers. In recent years, there has been an increase in oral cancer, which could be due to changes in sexual behavior in the general population. In México, there is scarce information on this regard, which prompted us to study HPV infection prevalence in the oral cavity of an indigenous community from the municipality of Siltepec, Chiapas, Mexico. Oral samples from 198 individuals were obtained with cytobrush for virus detection by nested PCR, using MY09/MY11 and GP5+/GP6+ primers, and positive samples were sequenced for HPV genotyping. In this study, 12.1% HPV infection prevalence was observed, which was shown to depend on gender, number of sexual partners, lack of using condoms, and oral sex practices. In contrast, no significant association between HPV infection and tobacco or alcohol consumption was observed. Furthermore, sequencing analyzes were performed where HPV-13 (21/24), -16 (2/24), -32 (1/24), -81 (1/24), and -83 (1/24) were observed and HPV-16 European/Asian and Asian/American E6 variants identified. These results evidenced an important prevalence of HPV infection in the oral cavity of a Mexican indigenous community, where the predominant genotypes were associated with benign pathologies, and demonstrated that high-risk genotype variants derived from different lineages.

Keywords: Oral cavity; human papillomavirus; genotypes; Mexican; nested PCR

1. Introduction

Human papillomavirus (HPV) belongs to a family of small non-enveloped virus with an 8-kb circular double-stranded DNA genome (Berbard *et al.*, 2010), which infects skin basal cells and mucosa squamous epithelium and has been associated with cervical cancer (CC), representing a major public health problem worldwide. In developing countries, CC is the most frequent female cancer, as well as the main cause of cancer-related death (Bruni *et al.*, 2015; Luna-Aguirre *et al.*, 2018). In Mexico, CC ranked second in incidence (16.9%) and mortality (11.9%) after breast cancer in 2012, among malignancies occurring in the female population. There are nearly 200 types of HPV, of which approximately 50 infect the genital area (Alemany *et al.*, 2015); mucosal HPV is classified as high and low risk depending on its association with neoplasia development. In addition to CC, about 30% to 40% of HPV-positive cases have been related to oral, oropharyngeal, penis, and vulva cancer (Muñoz *et al.*, 2006). In this regard, prevalence of high-risk HPV-16 and -18 in oral and oropharyngeal cancer has been reported (Pytynia *et al.*, 2014). Furthermore, HPV-16 was shown to be more frequent (>75%) and HPV-18 less frequent (<10%) in HPV-positive vulvar, vaginal, and anal carcinomas, as compared with CC (De Vuyst *et al.*, 2009).

During the past two decades, numerous studies have associated HPV infection with other epithelial neoplasias, such as head and neck squamous cell carcinoma (HNSCC) and oral squamous cell carcinoma (OSCC; HNSCC subtype), which constitutes 3% and 2% of all malignant neoplasms in males and females, respectively (D'Souza and Dempsey, 2011). Some evidence has linked them to orogenital contact with the transmission of

papillomavirus from the genital zone to the oral cavity. However, acquisition could be through mouth-to-mouth contact, autoinoculation, or independent events (Vigneswaran and Williams, 2014).

In recent decades, oropharynx cancer etiology has been associated with oncogenic HPV. In the United States the percentage of HPV positive-related oropharyngeal SCC and oropharyngeal tumors increased up to 82%; in Sweden, such an association increased up to 79% of tonsil SCC (Candotto, 2017). Recently, it was reported HPV-attributable fractions for the different HN subsites; HPV-attributable cancers amounted to 38,000 cases, whose relative HPV-16/-18 and HPV-6/-11/-16/-18/-31/-33/-45/-52/-58 contributions were 73% and 90%, respectively (Lobene, 1986).

In Mexico, there are few reports on the role of HPV infection in the oral cavity and it is very important to document the types and/or s HPV variants in different geographical regions. Provided that HPV promotes cervical, oral, and anal carcinogenesis, there is evidence that all these tumors can be preventable either with an efficient early diagnosis by detecting premalignant lesions, such as low and high grade intraepithelial lesions, accompanied by HPV detection or by broad HPV vaccination coverage to the population (women and men) (Schiffman *et al.*, 2007). Because of recent changes in sexual behavior in the general population (for example, initiation of sexual activity at an early age, increase in the number of sexual partners, and orogenital sexual practices), exposure to oral and genital infection occurs at early ages (Candotto, 2017).

It is important to determine HPV prevalence in the oral cavity of healthy people, and individuals with pre-neoplasic lesions or oropharyngeal carcinoma, to define prophylactic strategies. In particular, HPV detection and genotyping in the oral cavity

would provide a better understanding of its prevalence and diversity in the Mexican population, particularly from the municipality of Siltepec in Chiapas, Mexico.

2. Material and Methods

2.1 Study population

A cross-sectional study was developed in Siltepec, Chiapas, Mexico, which is a rural community located in the Sierra Madre of Chiapas, southwest Mexico (15º 33' N and 92º 20' W), with a population of around 38,145 inhabitants. The region is considered as a producer of migrants, with high levels of poverty and marginalization. In the present study, we included 198 individuals without previous clinical diagnosis of oral pathology, who voluntarily attended the dental brigade service during April 2017. Information about sociodemographic status, sexual behavior, clinical history, and tobacco and alcohol consumption were obtained through a direct interview with individuals. The presence of oral lesions was confirmed by oral clinicians, a maxillofacial surgeon, and a pathologist.

This study was approved by the scientific and bioethical committee of Centro de Investigación y Desarrollo en Ciencias de la Salud (CIDICS) and Dentistry Bio-ethics committee, Universidad Autónoma de Nuevo León (UANL), with approval SPSI-010613 number: 00132. A written informed consent was obtained from each participant.

Inclusion criteria included individuals (a) volunteering to participate in the study, (b) having signed the informed consent, (c) at least 18 years of age, and (d) attending the

dental brigade; exclusion criteria involved individuals (a) having been previously diagnosed with some type of cancer, (b) having current history of antimicrobials, antitumorals, and antivirals usage during the previous six months, and (c) presenting systemic diseases (autoimmunity and immunosuppression), hypertension, hormonal disorders, metabolic disorders, and psycho-motor disability.

2.2 Gingivitis evaluation

In this study, we used the noninvasive, no probing modified gingival index (MGI) to assess gingival inflammation in participants, which considers the following values: (a) normal (no inflammation), (b) mild inflammation (slight change in color, little change in texture of any portion of the gingival unit or inflammation of the entire gingival unit), (c) moderate inflammation (moderate glazing, redness, edema, and/or hypertrophy) of the entire gingival unit, and (d) severe inflammation (marked redness and edema/hypertrophy, spontaneous bleeding, or ulceration) of the gingival unit (Lobene, 1986).

2.3 Specimen and data collection

After oral clinical evaluation, oropharyngeal samples were collected by the exfoliative cytology scraping method, making a rotating movement with a cytobrush (Medical Packaging Co, Camarillo, CA) in different places of the oral cavity and pharyngeal such as the base of the tongue, inside of the cheeks, and hard and soft palate, excluding tonsils. Next, brushes were washed in a tube containing a preservative solution

(10 mM Tris-HCl, 1 mM NaCl, and 0.5% SDS, pH = 8) and samples were kept at -20 °C, until DNA extraction.

2.4 DNA extraction

For DNA extraction, samples were incubated with RNase (10 µg/mL) and allowed to incubate for 30 minutes at 37 ºC, followed by addition of proteinase K (1 mg/mL) for 1 hour at 56 ºC, and incubation for 10 minutes at 95 ºC for its inactivation. DNA was then purified using phenol-chloroform isoamyl alcohol technique and precipitated by salt/ethanol, as previously described (Sambrook *et al.*, 1989). Next, DNA was suspended in 50 µL TE buffer (0.01 M Tris HCl, pH 7.4, and 0.001 M EDTA) and incubated at 65 ºC for 1 hour, after which, DNA was stored at -80 °C, until analysis.

Extracted DNA integrity and adequacy were evaluated by detecting β-actin gene on PCR, using the oligonucleotides Fw 5'- GGCATCCTCACCCTGAAGTA-3' and Rv 5'-GGGGTGTTGAAGGTCTCAAA-3' as internal controls, electrophoresed in 8% acrylamide gel, stained with ethidium bromide, and visualized in an Ultraviolet Light Transilluminator Gel Doc™ XR+ Imager (Bio-Rad Lab). The expected PCR product was ~203 bp (Fig. 1).

2.5 HPV detection

HPV detection system uses MY09/MY11 and GP5+/GP6+ primers, that amplify the viral genome L1 region, which is frequently used for HPV detection in clinical and histological studies (Haws *et al.*, 2004). These primers are effective for amplifying wide spectrum of HPV genotypes in cells obtained from cervical smears and paraffin-embedded

tissues. MY09/MY11 primers flank a sequence of approximately 450 pb, whereas GP5+/GP6+ primers flank a sequence of about 150 pb, which is internal to the sequence flanked by MY (Snijders *et al.*, 1990). MY pair is synthesized from several degenerate nucleotides in each primer, it is a mixture of 25 oligonucleotides that amplify > 25 genital HPV types. On the other hand, GP5+/GP6+ primers consist of a fixed sequence of nucleotides, for each oligonucleotide primer.

HPV detection was performed by nested PCR, using MY09 5'-CGTCCMARRGGAWACTGATC-3' and MY11 5'-GCMCAGGGWCATAAYAATGG-3' primers, Taq DNA polymerase (5U/µL), dNTP mix, 100 ng DNA, and nuclease-free water for a final volume of 25 µL per reaction (MJ Mini Personal Thermal Cycler thermocycler; Bio-Rad Lab, Hercules, CA). Equipment programming was as follows: 1) Pre incubation at 95 °C for 3 min, 2) amplification at 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 90 s, for 40 cycles, and 3) extension at 72 °C for 4 min. The second amplification round was performed as the first one, but instead of adding DNA, 1 µL of first round PCR product was added and the primers used were GP5+ 5'-TTTGTTACTGTGGTAGATACTAC-3' and GP6+ 5'-GAAAAATAAACTGTAAATCATATTC-3' (Bio-Rad Lab). Equipment programming was as follows: 1) Pre incubation at 94 °C for 4 min, 2) amplification at 94 °C for 1 min, 40 °C for 2 min, and 72° C for 90 s, for 40 cycles, and 3) extension at 72 °C for 4 min. Next, PCR products were electrophoresed in a 8% acrylamide gel in 1X TBE buffer, then run at 90 Volts for 45 minutes, stained with ethidium bromide, and placed in an Ultraviolet Light Transilluminator Gel Doc™ XR+Imager (Bio-Rad Lab) to show ~150 bp amplicons, corresponding to HPV (Fig. 2).

2.6 L1 gene sequence-based HPV genotyping

HPV-positive samples were selected for HPV L1 genes molecular characterization by sequencing analysis. Nested PCR products were purified using a GeneJET NGS Cleanup Kit purification system (Thermo Scientific, Rockford, IL) and subjected to sequencing using the Big Dye Terminator Chemistry v3.1 Ready Reaction Kit (Applied Biosystems, Foster City, CA) and the GP6+ primer, in an automatic DNA sequencer (3500 Genetic Analyzer; Applied Biosystems, Foster City, CA), at Instituto de Fisiología Celular at Universidad Nacional Autónoma de México (Fig. 3).

Sequencing for each sample was performed in triplicate. DNA sequences were aligned and combined using the Bionumerics v7.1 program to construct a consensus DNA region that was compared with human papillomavirus known DNA sequences. Sequences and electropherograms were analyzed by Geneious v11.0.5 and FinchTV v1.4.0 programs.

The hypervariable region of the DNA sequence downstream of the GP5+ binding site is critical in L1 genotyping of HPV as the signature sequence. A nucleotide region of up to ~50 bp was selected and the identity analysis was carried out using the Basic Local Alignment Search Tool (BLAST) server (https://blast.ncbi.nlm.nih.gov/Blast.cgi), as previously reported by Lee *et al*. (2009).

2.7 Identification of nucleotide/amino acid changes and analysis of HPV-16 variants

HPV-16 E6 gene was amplified using the specific primers E6-F048 (5'-GAACCGAAACCGGTTAGTAT-3') and E6-R622 (5'-CAGTTGTCTCTGGTTGCAAA-3'), which amplify a region of ~575 bp. PCR was performed in a 50 µL reaction containing

1 μM of each oligonucleotide, 4 mM $MgCl_2$ and 1.25 U of high fidelity Platinum Taq DNA polymerase (Thermo-Scientific). Conventional PCR thermocycler was programmed as follows: initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 45 s, 57 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 10 min.

PCR products were purified using the GenJet NGS Cleanup Kit (Thermo-Scientific), following manufacturer's instructions. Products (both strands) were then sequenced using Big Dye Terminator Chemistry v3.1 Ready Reaction Kit on an automatic DNA sequencer (Applied Biosystems). Obtained sequences were aligned with HPV16R reference sequence (GenBank Accession NC_001526.4; Los Alamos National Laboratory, Los Alamos, NM). Using the E6 sequence, HPV-16 variants were classified into lineages with their respective sub-lineages, and sub-lineages were stratified into classes.

2.8 Statistical analysis

Differences in HPV prevalence for each group were assessed by contingency tables, and Chi-square and Fisher's exact tests were carried out for nominal variables; for numerical variables the Student *t* test was used. Statistical analyzes were performed using the SPSS v22 program.

3. Results

In this study, we enrolled 198 individuals. Relevant information (such as age, sex, tobacco and alcohol consumption, sexual behaviors, and family history) from each participant was collected by a specialist, using a questionnaire; oral pathologies, abnormalities in the oral cavity, and gingival state were also registered. Regarding sex and

age, 69.2% (137/198) were female and 30.8% (61/198) were male, whereas mean age was 41.8 ± 15.3 years old (mean ± SD), as shown in Table 1.

All samples, including DNA from HeLa cells, showed the expected ~203 bp β-actin gene amplicon (Fig. 1). In addition, in nested PCR for HPV detection, the expected ~150 bp amplicon was not observed in nuclease-free water, but in DNA from HeLa cells (Fig. 2). HPV positive individuals presented the ~150 bp DNA fragment in nested PCR.

HPV infection prevalence of 12.1% (24/198 of the subjects) was observed in the oral cavity. In addition, HPV infection percentage for females and males was 8.8% (12/137) and 19.7% (12/61), respectively. The mean age in individuals infected with HPV was 46.88 ± 17.89 years old and that of persons without infection was 41.12 ± 14.85 years old. No statistical association was observed between HPV infection and alcohol consumption and current tobacco smoking habits (Table 1). Furthermore, 62.5% (15/24) of individuals infected with HPV reported to be sexually active. A statistically significant (p = 0.001) association was found between oral HPV infection and the number of sexual partners, oral sex practices, and condom use; 58.4% (14/24) of the infected individuals reported equal or greater than 3 sexual partners. In addition, 79.2% (19/24) performed oral sex practices, 33.3% (8/24) reported never using condoms, 41.7% (10/24) sometimes using condoms, and 25% (6/24) always using condoms among infected individuals; the use of condoms was not for oral sex practices (Table 1). All individuals indicated to be heterosexual.

The relation of oral HPV infection and background of a relative diagnosed with head and neck squamous cell carcinomas (HNSCCs) was evaluated; 20.8% (5/24) reported having this background. Oral cavity lesions were identified in 11 individuals, only 2

individuals were infected presented oral lesions (one case of condyloma acuminatum and one case of MEH), as shown in Table 1. Furthemore, a statistically significant (p = 0.001) association between gingival status and oral HPV infection was observed; 58.3% (14/24) and 37.5% (9/24) of infected individuals presented mild and moderate inflammation, respectively. Only one individual infected with HPV did not present gingival inflammation (Table 1).

Electropherograms were analyzed and sequences alignment allowed identification of types 13, 16, 32, 81, and 83. On the other hand, genotype 18 was detected in HeLa cells positive control. Electropherograms, alignments, and identity analysis are shown in Figures 4 and 5. Based on genotyping results, the most frequent type was HPV-13 with 87.5% (21/24), followed by HPV-16 with 8.33% (2/24), and HPV- 32, HPV-81, and HPV-83 with 4.17% (1/24) each; co-infection of genotypes HPV-13 / HPV-16 and HPV-13 / HPV-81 was also observed (Table 2).

For the analysis of variants, E6 HPV-16 sequence was amplified using type-specific oligonucleotides and subsequent amplicon sequencing ~575 bp. Analysis of variants was developed for HPV-16 genotype positive samples. HPV16R sequence was used as a reference for the alignment of the sequences obtained from electropherograms. In one of the samples, the following nucleotide changes were observed: G131 (from adenine to guanine at position 131) and G350 (from thymine to guanine at position 350). These nucleotide changes led to the following amino acid changes: R10G (from arginine to glycine at position 10) and L83V (from leucine to valine in position 83). The sample was phylogenetically classified as variant E-G131/G350 (European-Asian lineage/European sub-lineage/Class E-G350).

In the other sample, the following nucleotide changes were observed: T145 (from guanine to thymine at position 145), A286 (from thymine to adenine at position 286), G289 (from adenine to guanine at position 289), T335 (from cytosine to thymine at position 335), G350 (from thymine to guanine at position 350), and G532 (from adenine to guanine at position 532). Of these changes, only three produced the amino acid changes Q14H (from glutamine to histidine at position 14), H78Y (from histidine to tyrosine at position 78), and L83V (from leucine to valine at position 83). The sample was phylogenetically classified as AA-a variant (Asian American/North American lineage/Asian American sub-lineage/class AA-a).

4. Discussion

Worldwide researchers have reported the presence of human papillomavirus in different areas of the human body, including the oral cavity, where infection has been recently associated with the development of cancer. Recently, anti-HPV immunization was introduced in some countries, including Mexico (Luciani et al., 2018). The strategy, proposed by Mexico, includes primary prevention for girls between 9 and 16 years old. However, studies in different regions of Mexico have frequently detected other genotypes that are not included in this scheme. In addition, existing vaccines were designed for prevention of precancerous lesions and SCC in the anogenital area, thus their effectiveness in OSCC prevention is still unknown.

In Mexico, there are few reports on the role of HPV infection in the oral cavity and its implications in the general population, and it is very important to document the types

and/or variants of HPV in different geographical regions. The aim of the present study was to investigate the presence of HPV in the oral mucosa of indigenous individuals from Chiapas, Mexico. Several studies have demonstrated higher sensitivity and specificity of nested PCR system using the two oligonucleotide pairs MY09/11 and GP5+/GP6+, compared with other methods (Cai *et al.*, 2013; Entiauspe *et al.*, 2013), which allows for proper identification of any HPV type, present in the sample.

In our study, there was no significant difference in the mean age of individuals infected or not with HPV, and between HPV infection and drinking habits (p = 0.918) or tobacco use (Table 1). These results agreed with those obtained by Ibieta-Zarco *et al.* (2005), who did not observe a statistically significant association between these variables, in patients diagnosed with HNSCCs. Gonzalez-Losa *et al.* (2015) conducted an epidemiological study in women without oral pathology symptoms, whose results agreed with those of the present work. On the other hand, Kreimer *et al.* (2010), in a multi-national study including Mexico, found that tobacco consumption is a factor strongly related to oral HPV infection in asymptomatic individuals.

In addition, a statistically significant association was found between HPV infection and different sexual behaviors, such as higher number of couples throughout their lives, oral sex practices, and sex without a condom (Table 1); this observation agrees with several studies showing such factors as potential risks to HPV infection (Smith *et al.*, 2004). This could be related to the main mechanism of transmission of the virus (sexual transmission through fluids and warts), suggesting that the use of condoms during oral sex practices could reduce the rate of oral infection. Furthermore, a statistically significant association between the gender and HPV infection was observed (Table 1). Kreimer *et al.* (2010)

conducted a study in healthy individuals, finding a similar prevalence of oral HPV in men and women (4.6% and 4.4%, respectively).

We also demonstrated the presence of viral HPV DNA in 12.1% of individuals between 18 and 72 years old from Siltepec community, which indicates a significant prevalence of the infection in this population. In this regard, Gonzalez-Losa *et al.* (2015, 2013), reported a prevalence of about 14% in individuals without symptoms of apparent oral disease from Yucatan, Mexico. In addition, Kreimer *et al.* (2010), included 591 healthy Mexican men, observing a prevalence of 5.9%, of which 1% corresponded to high-risk (HR) types, mainly HPV-16. It is possible that HPV detection in oral cavity samples could be used as a tool for the diagnosis of HPV types related to the development of oral pathologies.

Among individuals infected, 91.7% single-type infection and 8.3% multiple-type infection were observed. For HPV-16 (considered a high-risk genotype), one case was identified in both single-type and multiple-type infection. Overall, HPV-13 was the most prevalent with 87.5% (n =21), as shown in Table 2. These data agreed with those reported by Jiménez *et al.* (2001), who observed 90.09% of LR genotypes (HPV-6, -13, and -32), where 45.45% corresponded to genotype 13, and 9% of the samples presented mixed infection.

HPV-13 was the genotype with the highest prevalence in our population (Table 2). This genotype is strongly associated with multifocal epithelial hyperplasia (MEH), which is a rare pathology of the oral mucosa that has been described in some ethnic groups, mainly in the Americas (Cuberos *et al.*, 2006). Several studies have reported the existence of this pathology in Mayan locations in southern Mexico (Gonzalez-Losa *et al.*, 2013, 2011;

Lopez-Villanueva *et al.*, 2011); it is characterized by asymptomatic lesions that can develop in any part of the oral mucosa, but more frequently at the level of the tongue, soft palate, and lips. The presence of this viral genotype in the oral mucosa has also been described in individuals without apparent lesions (Gonzalez-Losa *et al.*, 2013; Cuberos *et al.*, 2006; Lopez-Villanueva *et al.*, 2011). MEH typically occurs during the first decade of life. Asymptomatic infection in women between 16 and 40 years of age could play a role in the transmission and maintenance of this virus in the community and within families. These results suggest the need for longitudinal studies to increase our knowledge of the dynamics of infection by HPV-13, which was considered the exclusive genotype of the oral cavity; however, recent studies have reported that it can also be found in the genital tract. Illades-Aguilar *et al.* (2010) and Mattos *et al.* (2011) reported a prevalence of 0.1% and 4.3% of this genotype in the cervix, respectively. These low percentages of HPV-13 could be partially explained by the fact that several HPV genotyping techniques cannot determine this type or because of the unusual geographical distribution of this genotype. In this concern, Lopez-Villanueva *et al.* (2011) reported the presence of HPV-13 in all saliva samples in individuals with MEH from Mérida, Yucatán, which could be explained by the domestic transmission of HPV-13 through saliva and the shared use of contaminated objects. Another genotype that is associated with MEH is HPV-32, however, this genotype is frequently detected in the genital tract. In our study, HPV-13 was the most frequent genotype, but one of the individuals (1/10) who presented MEH was infected with HPV-32 genotype. This discrepancy could suggest that MEH lesions observed in our population could be attributed to other factors related to this type of lesions, including genetic predisposition, nutritional deficiencies, environmental factors, and immunosuppression (Bascones-Martinez *et al.*, 2012). On the other hand, HPV-16 genotype in the oral cavity of

2 individuals and genotypes HPV-32, -81, and -83 in 1 patient each were observed. These results differ from those observed by Gonzalez-Losa *et al.* (2011), who identified HPV-6, -58, -16, -81, -59, and -18 genotypes.

In addition, according to the sequencing analysis, E6 gene nucleotide variants were detected and used them to investigate HPV heterogeneity in our population. It is known that there are multiple HPV-16 variants which are distributed worldwide, thus the risk of developing SCC may be higher in some populations than in others. In the present study, one of the samples was classified as variant E-G131/G350, which belongs to the European-Asian lineage, European sub-lineage, and Class E-G350, and the other as AA-a variant of Asian American/North American lineage, Asian American sub-lineage, and AA-a class. Ortiz-Ortiz *et al.* (2015) reported 27 HPV variants in 330 women infected with HPV-16 from the state of Guerrero, Mexico; 82.12% belonged to variants of the European sub-lineage, 17.58% to Asian American sub-lineage 1, and 0.3% to African sub-lineage 2a. The most common one was E-G350 (40%), followed by E-prototype (13.03%), E-C188/G350 (11.82%), AA-a (10.61%), AA-c (6.07%), and E- A176/G350 (5.15%).

It is known that the genomes of HPV-16 variants geographically differ worldwide, because of the evolution related to ethnic groups and that the risk of cervical carcinoma appears to be population dependent (Yamada *et al.*, 1997; Zuna *et al.*, 2009). Mexico is a country with diverse ethnic origins because European immigrants mixed with several indigenous populations, consequently the current population carries the HPV variant of several ethnic groups (Calleja-Macias *et al.*, 2004).

Our results evidenced an important prevalence of HPV infection in the oral cavity of a Mexican indigenous community, where the predominant genotypes were associated with

benign pathologies, and demonstrated that high-risk genotype variants derived from different lineages.

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Figure Legends

Figure 1. PCR amplifications for the β-actin gene. M, HyperLadder ™ 100bp (Bioline, Tauton, MA); 1, negative control (nuclease-free water); 2, positive control (HeLa cell line DNA with HPV-18); 3-9, DNA from individual samples, in 8% acrylamide gel.

Figure 2. Amplifications of nested PCR using GP5+/GP6+ oligonucleotides for the HPV L1 gene ~150 bp region. M, HyperLadder ™ 100bp (Bioline); 1, negative control (nuclease-free water); 2, positive control (HeLa cell line DNA with HPV-18); 3-4, DNA from negative individuals; 5-8, DNA from positive individuals. in 8% acrylamide gel.

Figure 3. Purified ~150 bp amplicons. M, HyperLadder ™ 100bp (Bioline); 1-24, positive by nested PCR; 25, positive control (HeLa cell line DNA with HPV-18), in 1.5% agarose gel.

Figure 4. Electropherograms, alignments, and identity analysis. a) HPV-16, b) HPV-13, and c) HPV-81.

Figure 5. Electropherograms, alignments, and identity analysis. a) HPV-18 (control of the HeLa cell line), b) HPV-32 and c) HPV-83.

Table 1. Distribution of HPV infection in the oral cavity of individuals by age, gender, habits, sexual behavior, family history, oral cavity lesions, and gingival status

	HPV (nested PCR)			
	Total (n=198)	**Positive (n=24)**	**Negative (n=174)**	***p* value**
Gender	**n (%)**	**n (%)**	**n (%)**	
Female	137 (69.2)	12 (50.0)	125 (71.8)	0.0298[a]
Male	61 (30.8)	12 (50.0)	49 (28.2)	
Age (mean±SD)	41.82±15.32	46.88±17.89	41.12±14.85	0.085[b]
Alcohol [d]				
Yes	56 (28.3)	7 (29.2)	49 (28.2)	0.918[a]
<20	40 (20.2)	4 (16.6)	36 (20.7)	
>20	16 (8.1)	3 (12.6)	13 (7.5)	
Never	142 (71.7)	17 (70.8)	125 (71.8)	
Tobacco [e]				
Yes	13 (6.6)	3 (12.5)	10 (5.7)	0.198[c]
<10	10 (5.1)	0	10 (5.7)	
>10	3 (1.5)	3 (12.5)	0	
Never	185 (93.4)	21 (87.5)	164 (94.3)	
Sexual activity				
Yes	146 (73.7)	15 (62.5)	131 (75.3)	0.182[a]
No	52 (26.3)	9 (37.5)	43 (24.7)	
Total number of sexual partners				
1	162 (81.8)	5 (20.8)	157 (90.2)	0.001[a]
2	20 (10.1)	5 (20.8)	15 (8.6)	
≥ 3	16 (8.1)	14 (58.4)	2 (1.2)	
Oral sex practices ever				
Yes	21 (10.6)	19 (79.2)	2 (1.2)	0.001[c]
No	177 (89.4)	5 (20.8)	172 (98.8)	
Condom use				
Never	23 (11.6)	8 (33.3)	15 (8.6)	0.001[a]
Occasionally	75 (37.9)	10 (41.7)	65 (37.4)	
Always	100 (50.5)	6 (25.0)	94 (54.0)	

Family member diagnosed with head and neck cancer				
Yes	15 (7.6)	5 (20.8)	10 (5.7)	0.023[c]
No	183 (92.4)	19 (79.2)	164 (94.3)	
Oral cavity lesions				
Yes	11 (5.6)	2 (8.3)	9 (5.2)	0.627 [c]
Condyloma acuminatum	1 (0.5)	1 (4.15)	0	
MEH	10 (5.1)	1 (4.15)	9 (5.2)	
No	187 (94.4)	22 (91.7)	165 (94.8)	
Modified Gingival Index[f]				
Without inflammation	123 (62.1)	1 (4.2)	122 (70.1)	0.001 [c]
Mild inflammation	65 (32.8)	14 (58.3)	51 (29.3)	
Moderate inflammation	10 (5.1)	9 (37.5)	1 (0.6)	

[a]Chi-squared test, [b]Student *t* test, [c]Fisher's exact test, [d]Mean drinks/week, [e]Mean cigarettes/day (current smokers), [f]MGI is a non-invasive method that allowed to know the inflammatory state of the gingiva without the use of a probe.

Table 2. HPV genotype frequencies and percentages

HPV types	**HPV-positive patients (%)**
Single type	**22 (91.7)**
HPV-13[a]	19 (79.20)
HPV-16[a]	1 (4.16)
HPV-32	1 (4.16)
HPV-83	1 (4.16)
Multiple type	**2 (8.3)**
HPV-13/HPV-16	1 (4.16)
HPV-13/HPV-81[a]	1 (4.16)

[a]The overall prevalence for HPV-13, -16, and -81 was 87.5% (n =21), 8.32% (n =2), and 4.16% (n =1), respectively.

Myriam Angélica de la Garza-Ramos: Conceptualization, Supervision, Methodology, Resources, Funding acquisition: **Victor Hugo Urrutia-Baca**: Methodology, Software, Investigation, Formal analysis, Writing; **Cynthia Sofia Urbina-Rios:** Validation, Writing, Investigation; **Dabeiba Adriana García Robayo**: Validation, Writing; **Patricia Tamez-Guerra**: Validation, Writing; **Ricardo Gomez-Flores**: Conceptualization, Supervisión, Writing – Review & Editing, Visualization

DECLARATIONS OF INTERESTS: NONE

15°33’N 92°20’W

Indigenous community → HPV prevalence → HPV-16 European/ Asian and Asian/ American E6 variants

Highlights

- Prevalence of HPV infection in the oral cavity of a Mexican indigenous community
- Predominant genotypes were associated with benign pathologies
- High-risk genotype variants derived from different lineages

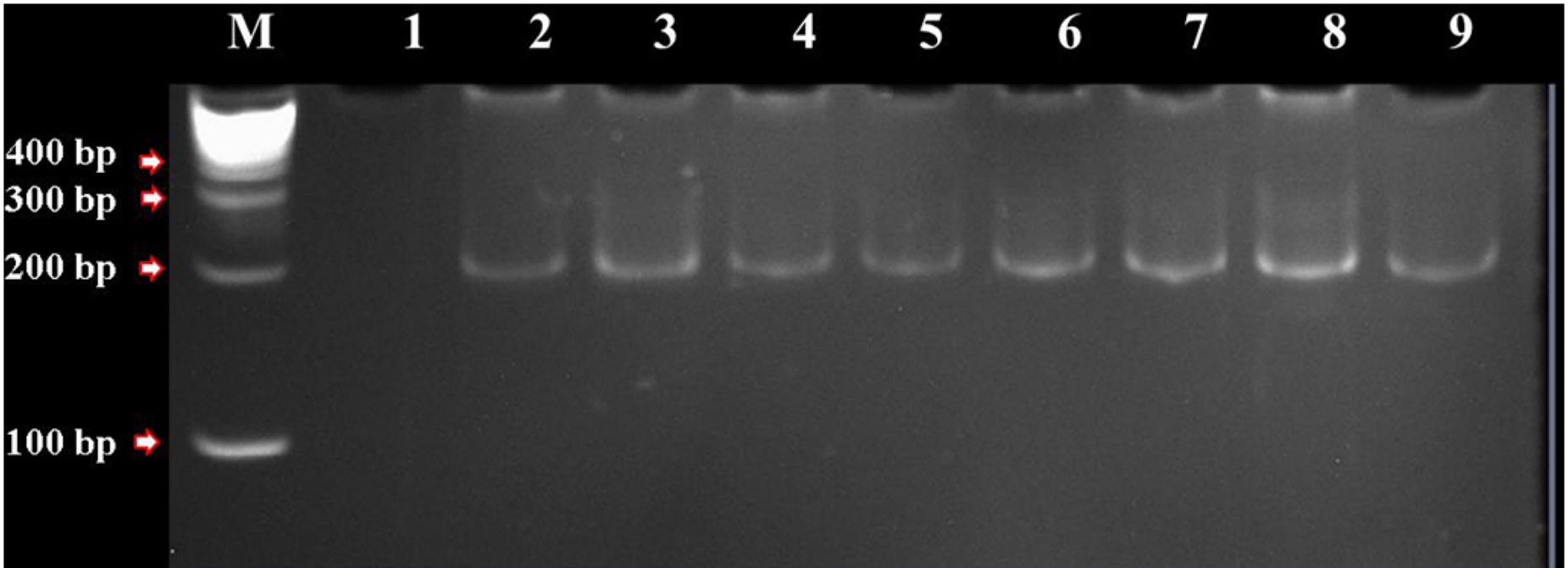


Figure 1

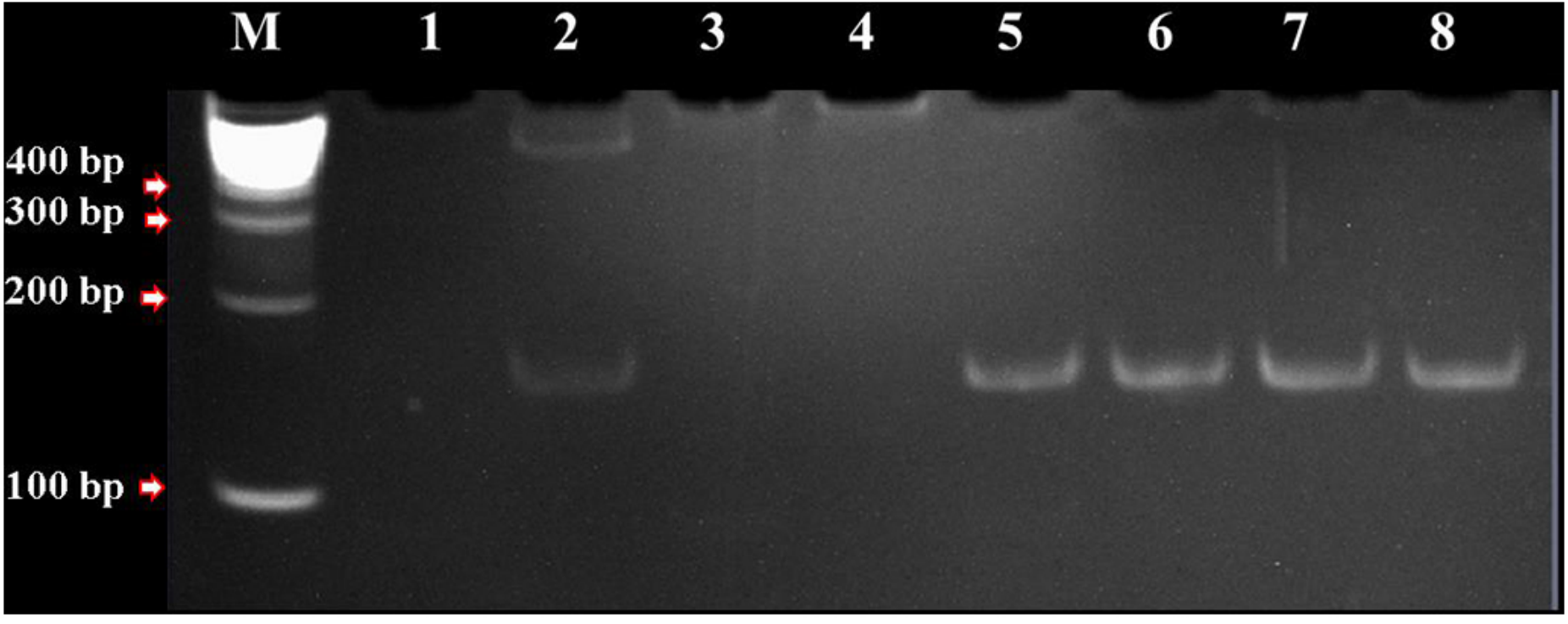


Figure 2

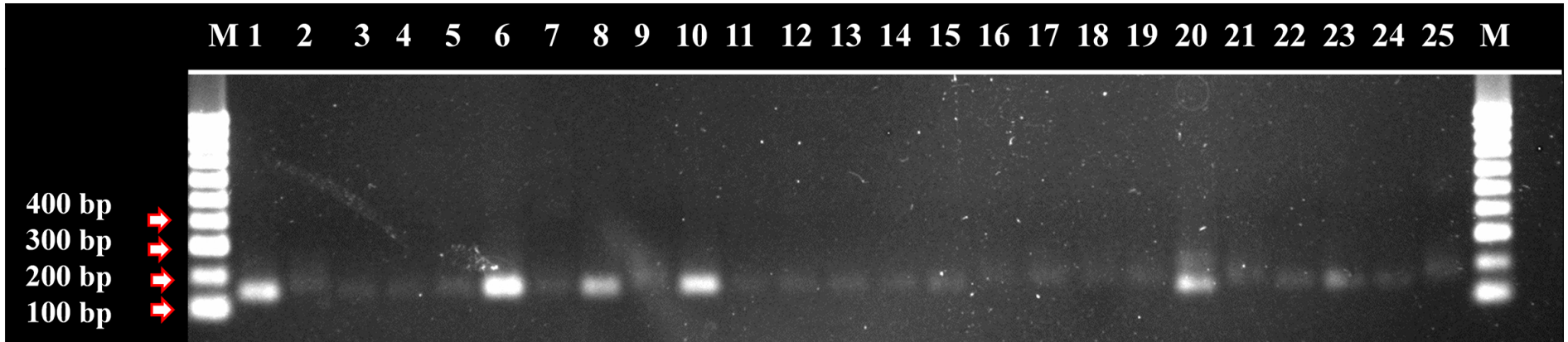

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 M
400 bp
300 bp
200 bp
100 bp

Figure 3

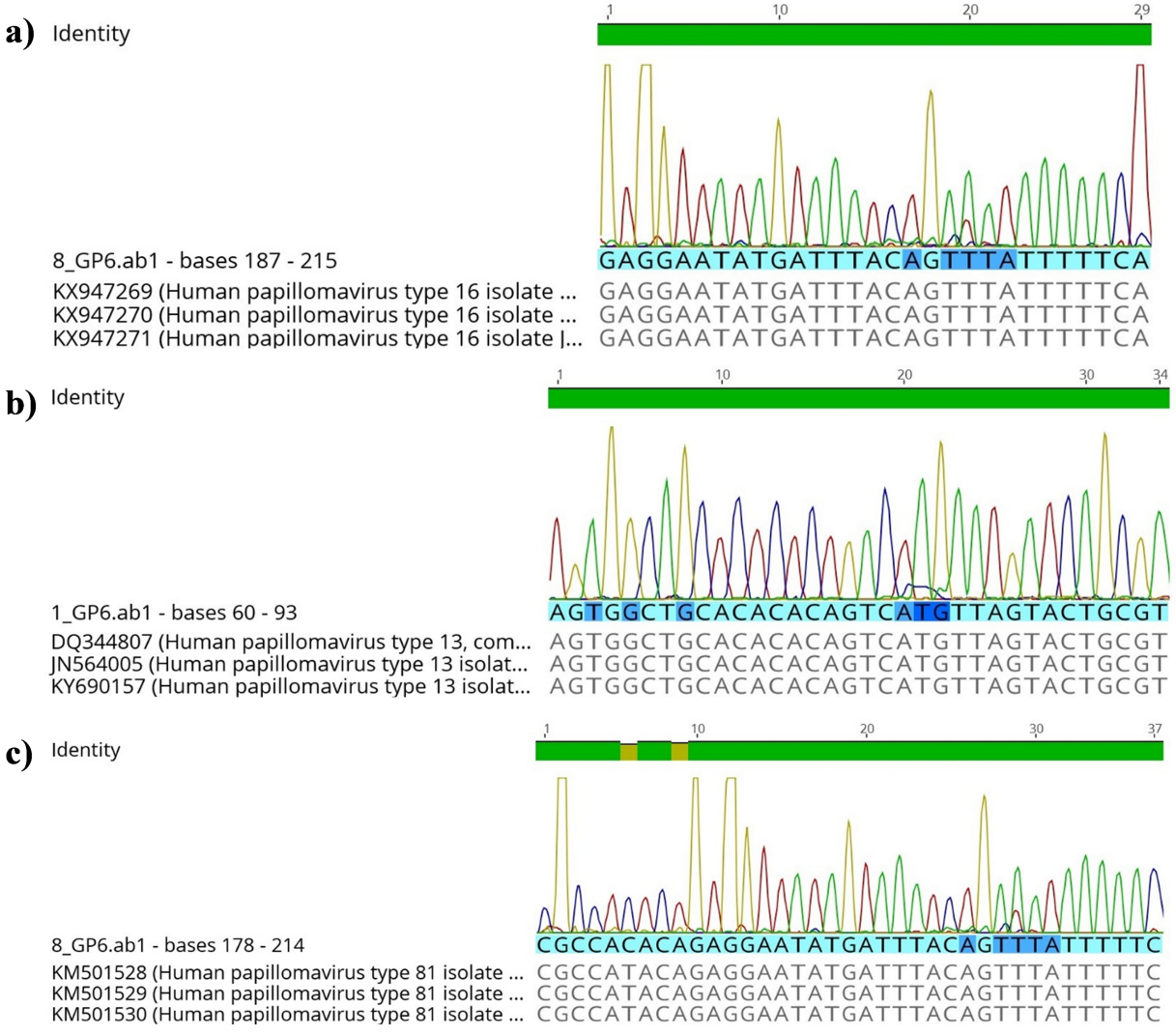
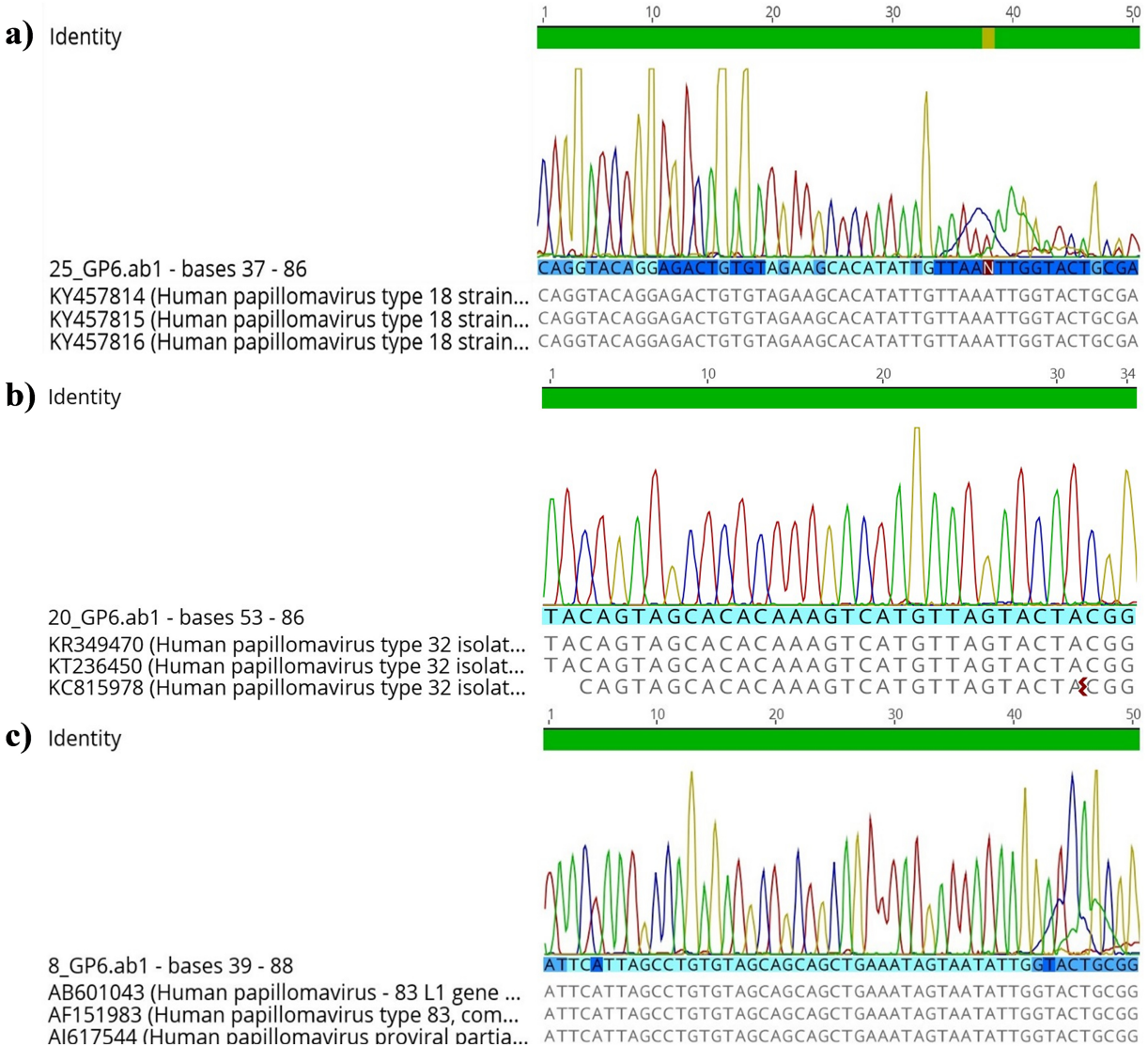

a)
Identity
1
10
20
29
8_GP6.ab1 - bases 187 - 215
GAGGAATATGATTTACAGTTTATTTTTCA
KX947269 (Human papillomavirus type 16 isolate ...
KX947270 (Human papillomavirus type 16 isolate ...
KX947271 (Human papillomavirus type 16 isolate ...
GAGGAATATGATTTACAGTTTATTTTTCA
b)
Identity
1
10
20
30
34
1_GP6.ab1 - bases 60 - 93
AGTGGCTGCACACACAGTCATGTTAGTACTGCGT
DQ344807 (Human papillomavirus type 13, com...
JN564005 (Human papillomavirus type 13 isolat...
KY690157 (Human papillomavirus type 13 isolat...
AGTGGCTGCACACACAGTCATGTTAGTACTGCGT
c)
Identity
1
10
20
30
37
8_GP6.ab1 - bases 178 - 214
CGCCACACAGAGGAATATGATTTACAGTTTATTTTTC
KM501528 (Human papillomavirus type 81 isolate ...
KM501529 (Human papillomavirus type 81 isolate ...
KM501530 (Human papillomavirus type 81 isolate ...
CGCCATACAGAGGAATATGATTTACAGTTTATTTTTC

Figure 4


a)
Identity
1
10
20
30
40
50
25_GP6.ab1 - bases 37 - 86
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KY457814 (Human papillomavirus type 18 strain...
CAGGTACAGGAGACTGTGTAGAAGCACATATTGTTAAATTGGTACTGCGA
KY457815 (Human papillomavirus type 18 strain...
CAGGTACAGGAGACTGTGTAGAAGCACATATTGTTAAATTGGTACTGCGA
KY457816 (Human papillomavirus type 18 strain...
CAGGTACAGGAGACTGTGTAGAAGCACATATTGTTAAATTGGTACTGCGA
b)
Identity
1
10
20
30
34
20_GP6.ab1 - bases 53 - 86
TACAGTAGCACACAAAGTCATGTTAGTACTACGG
KR349470 (Human papillomavirus type 32 isolat...
TACAGTAGCACACAAAGTCATGTTAGTACTACGG
KT236450 (Human papillomavirus type 32 isolat...
TACAGTAGCACACAAAGTCATGTTAGTACTACGG
KC815978 (Human papillomavirus type 32 isolat...
CAGTAGCACACAAAGTCATGTTAGTACTACGG
c)
Identity
1
10
20
30
40
50
8_GP6.ab1 - bases 39 - 88
ATTCATTAGCCTGTGTAGCAGCAGCTGAAATAGTAATATTGGTACTGCGG
AB601043 (Human papillomavirus - 83 L1 gene ...
ATTCATTAGCCTGTGTAGCAGCAGCTGAAATAGTAATATTGGTACTGCGG
AF151983 (Human papillomavirus type 83, com...
ATTCATTAGCCTGTGTAGCAGCAGCTGAAATAGTAATATTGGTACTGCGG
AJ617544 (Human papillomavirus proviral partia...
ATTCATTAGCCTGTGTAGCAGCAGCTGAAATAGTAATATTGGTACTGCGG

Figure 5