

A pilot study on salivary HPV DNA detection to monitor active disease from patients with recurrent respiratory papillomatosis

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ABSTRACT

Purpose: Recurrent respiratory papillomatosis (RRP) is a human papillomavirus (HPV)-related disease affecting the upper airway and saliva could be an important non-invasive sampling source for viral screening and clinical monitoring. We investigated whether HPV DNA could be detected in saliva (cellular pellets and supernatant) from RRP patients and influence on clinical manifestation of the disease.

Materials and methods: In this pilot study, saliva samples from 14 RRP patients were obtained in preoperative condition (n = 7) and in disease-free interval (DFI; n = 7). Healthy donors (n = 14) were also included. HPV DNA was investigated by polymerase chain reaction (PCR)-based assays.

Results: From cellular pellets, HPV-positive saliva was only detected from preoperative collections (5/7; 71.4 %) and showed a mean cycle threshold (Ct) value of 24.33 (±1.25), whereas all patients in DFI were HPV-negative (Ct ≥ 32.16), revealing significant difference between these two clinical moments (p = 0.021). Patients in DFI and healthy donors showed similar Ct values. From saliva supernatant, detectable HPV cell-free DNA (cfDNA) occurred in 42.9 % (3/7) and 57.1 % (4/7) of preoperative collections using the commercial cfDNA kits from Norgen and Qiagen, respectively. Salivary cfDNA size distribution obtained by TapeStation analysis showed a predominant size range of 150 to 400 bp in both patients and healthy controls, corresponding to mono-nucleosomal and dinucleosomal fragments.

Conclusions: In conclusion, HPV DNA screening in saliva (both cellular pellets and cfDNA) may have clinical utility to monitor active disease of RRP patients.

1. Introduction

Recurrent respiratory papillomatosis (RRP) is a chronic human papillomavirus (HPV)-related disease characterized by papillomatous lesions in the upper airway, usually caused by HPV-6 and/or HPV-11 infection in larynx. Both children and adults are affected by the disease, which shows a high tendency of recurrence and leads to repeated surgeries lifelong to avoid respiratory obstruction by the papillomatous lesions [1,2]. RRP with an aggressive clinical course can be described by multiple surgical interventions *per* year, tracheostomy need, papillomatous lesions detected in extra-laryngeal sites, and malignant transformation [2–4]. Molecular tests would be an important strategy to track RRP patients with active disease and to better stratify those at risk of an

aggressive clinical course of the disease. Since RRP affects the upper airway, saliva can be a suitable and non-invasive sampling source for clinical monitoring of the patients. Furthermore, saliva can be used in several pathologies for viral detection, including screening of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), Epstein-Barr virus (EBV), and HPV [5–8].

Liquid biopsy is an emerging investigation area based on minimally invasive procedures with potential to track molecular alterations clinically useful through biological fluids (e.g., blood, saliva, and urine) [9,10]. One of the most investigated molecular category in liquid biopsy is represented by the cell-free DNA (cfDNA), usually released into bio-fluids from processes of cell death (e.g., apoptosis, necrosis, and autophagy) or active cellular secretions [11,12]. HPV cfDNA screening in

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biofluids has been reported as a diagnostic and prognostic biomarker useful for clinical surveillance and to describe the disease status of HPV-associated oropharyngeal cancer [13,14].

The HPV viral load dynamics can be assessed by polymerase chain reaction (PCR) assays through the cycle threshold (Ct) values. The Cobas® 4800 HPV Test (Roche Molecular Systems, Pleasanton, CA, USA) is an automated system clinically FDA-approved based on PCR technology to detect 14 high-risk HPV (HR-HPV) types. Cobas® test has been used to better risk stratification of cervical cancer and cervical precursor lesions through Ct values, in which smaller Ct values represent higher viral copies [15,16]. In this work, we investigated whether HPV DNA can be detected in saliva (both cellular pellets and circulating cfDNA) from RRP patients to evaluate the influence of the HPV viral load dynamics on clinical manifestation of the disease.

2. Material and methods

2.1. Study participants

This study included 28 participants, being 14 RRP patients and 14 healthy donors (without HPV history), recruited between March 2020 and November 2021. Patients had clinical and anatomopathological diagnosis of RRP and were treated at the Head and Neck Surgery and Respiratory Endoscopy Service of the Federal Hospital of Bonsucesso (HFB) and at the Otorhinolaryngology Service of the Pedro Ernesto University Hospital (HUPE), both in Rio de Janeiro, Brazil. None of the patients in this study had received the HPV vaccine prior to RRP diagnosis, not even the mothers of the juvenile patients. Both adult onset RRP (AoRRP $\geq$ 18 years old) and juvenile onset RRP (JoRRP $<$ 18 years old) were eligible for participation. Patients with a previous history of immunosuppression, like those using immunosuppressive drugs and/or with chronic immunodeficiency disorders, were excluded from this study. This study was approved by the Ethics and Research Committee of both hospitals: HUPE (CAAE:30816114.8.0000.5259) and HFB (CAAE:26539019.6.0000.5253). Written informed consent was obtained from each healthy donor, adult patient, and legal guardian of pediatric patients.

2.2. Saliva collection and processing

Unstimulated saliva samples (~2 mL) from all participants were collected into a sterile 50 mL tubes and saliva samples were immediately placed on ice until further processing within 2 h of collection. For RRP patients, saliva collection occurred into two different clinical conditions: 1) 1 h before the surgical intervention (preoperative collection; $n = 7$); 2) during the disease-free interval (DFI; ≥ 2 months since last surgery; $n = 7$).

Saliva samples were centrifuged at 3000 $\times g$ for 20 min at 4 °C. Both cellular pellets and supernatants were used for DNA extraction. Samples were aliquoted and stored at -80 °C until further analysis.

2.3. DNA isolation from salivary cellular pellets and HPV detection by conventional polymerase chain reaction (PCR)

For DNA extraction, cellular pellets were resuspended in proteinase K solution and lysis buffer (10 mM Tris-HCl, 25 mM EDTA, 100 mM NaCl, and 1 % SDS) followed by the phenol-chloroform method, according to standard protocols [17]. DNA was stored at -20 °C until further analysis. DNA from each sample was quantified in the Qubit® fluorometer 3.0 (Invitrogen, Life Technologies) by using the Qubit dsDNA HS Assay Kit (Invitrogen), according to the manufacturer's protocol.

HPV DNA detection (PGMY09/11) and genotyping (HPV-6, HPV-11, HPV-16, and HPV-18) were assessed by conventional PCR technique, as described in our previous study [18]. PCR products (PGMY09/11: 450 bp; HPV-6: 280 bp; HPV-11: 360 bp; HPV-16: 246 bp; HPV-18:162 bp)

were visualized on nondenaturing 10 % polyacrylamide gels stained with silver.

2.4. Cycle threshold (Ct) values of HPV by quantitative PCR (qPCR)

HPV-Ct values for PGMY09/11, HPV-6, and HPV-11 detection were obtained by qPCR reactions to compare the differences in HPV viral load in salivary cellular pellets. The qPCR reactions were performed in a final volume of 25 μ L using DNA samples (100 ng), primers sequences [18], and Power SYBR™ Green PCR Master Mix (Life Technologies, Carlsbad, CA) in platform StepOnePlus™ Real-Time PCR System (Life Technologies, Carlsbad, CA). The qPCR reactions were carried out in duplicate. The amplification of human β -globin gene was used as an internal control.

For the standard curve, serial dilutions with 10-fold dilutions (100 to 0.1 ng of DNA) were performed from saliva sample of Pt. 02 and the Ct values were plotted against the ng of DNA used as input *per* reaction.

2.5. Isolation and quantification of salivary cfDNA

CfDNA was isolated from 1 mL of saliva supernatant using the QIAamp Circulating Nucleic Acid Kit (Cat. # 55114; Qiagen, Hilden, Germany) and the Plasma/Serum Circulating Nucleic Acid Purification Mini Kit (Slurry Format) (Cat. # 53300; Norgen Biotek, Canada). Both commercial kits were used according to the manufacturer's instructions. Isolated salivary cfDNA was stored at -80 °C until further analysis.

Quantification of salivary cfDNA was performed in the Qubit® fluorometer 3.0 (Invitrogen, Life Technologies) by using the Qubit dsDNA HS Assay Kit (Invitrogen), according to the manufacturer's protocol. The cfDNA fragment size was assessed with the Agilent 4200 TapeStation System (Agilent Technologies, Santa Clara, California) using the D1000 ScreenTape assay (Agilent Technologies).

2.6. Detection of salivary HPV cfDNA by cycle threshold (Ct) values

Since the cfDNA fragment size is shorter than the cellular DNA [19], qPCR assays were performed by using primers sequences that yield shorter amplicon length to detect HPV-6 (80 bp) and HPV-11 (112 bp) [20]. The amount of cfDNA input for each qPCR reaction was nearly 20 ng. The qPCR reactions (final volume = 25 μ L) using Power SYBR™ Green PCR Master Mix (Life Technologies, Carlsbad, CA) were performed in platform StepOnePlus™ Real-Time PCR System (Life Technologies, Carlsbad, CA). All qPCR assays were carried out in duplicate.

2.7. Statistical analysis

Statistical analyses for the association between salivary HPV DNA detection and Ct values and for the differences in cfDNA concentrations between patients and controls were evaluated using Fisher's exact test with the GraphPad Prism 9 (GraphPad Software version 9.3.1). For all statistical analyses, a level of significance of 5 % was considered ($p < 0.05$).

3. Results

3.1. Characteristics of the participants

Fig. 1 represents the schematic workflow from saliva collection to HPV detection assays. This study included 14 RRP patients (female = 5; male = 9) and 14 healthy controls (female = 8; male = 6). RRP patients were represented by 9 AoRRP (mean age: 46.56 years; range: 21 to 72) and 5 JoRRP (mean age: 9.2 years; range: 03 to 17). The mean age of healthy controls was 34.43 years (range: 17 to 59). Regarding HPV detection in biopsy specimens from the study RRP patients, 57.1 % (8/14) carried HPV-6, followed by 28.6 % (4/14) of HPV-11 and 14.3 % (2/14) of HPV-6/11 coinfection, with no HPV-16 or HPV-18 detection. The

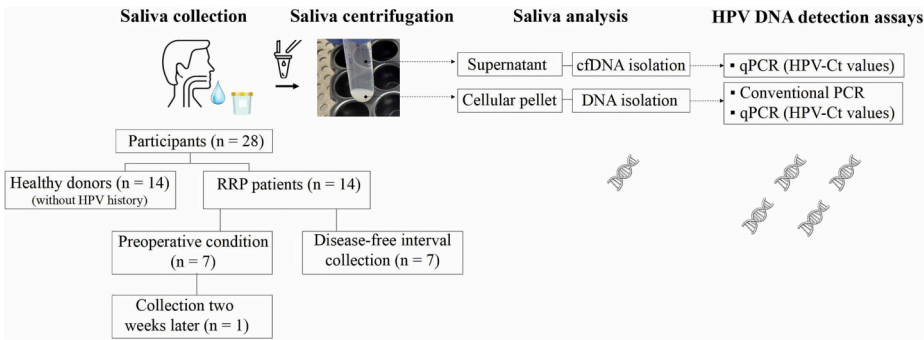


Fig. 1. Schematic representation of the workflow for salivary HPV detection. Twenty-eight participants were invited to this study. Unstimulated saliva samples were collected into sterile tubes. Both salivary cellular pellets and supernatant were analyzed by PCR-based assays to detect HPV DNA.

HPV type carried by patients in the disease-free interval (DFI) had already been recorded in our previous studies. All these characteristics are listed in Table 1.

In this study, most of saliva collections were carried out during the pandemic situation of SARS-CoV-2 infection. Therefore, we asked whether the participants had been infected with SARS-CoV-2 prior to saliva collection, since it could deregulate the immunity and thus influence in HPV detection. By self-report, we recorded that none of participants was previously infected with SARS-CoV-2 (Table 1).

3.2. HPV DNA detection in salivary cellular pellets by conventional PCR

Using PGMY09/11 primer set, PCR products on polyacrylamide gels revealed that HPV DNA was only detectable in salivary cellular pellets from RRP patients compared with healthy controls. Among RRP patients, HPV DNA was only found in saliva from preoperative collections (5/7; 71.4 %). For HPV-positive saliva, HPV genotyping revealed detection of HPV-6 or HPV-11, corresponding to the same HPV type detected in biopsy specimens. It is worthwhile reporting that the two saliva samples from preoperative collection with undetectable HPV DNA yielded insufficient quantity of cellular pellet after centrifugation compared with all other study saliva samples, which may have affected the quality of HPV detection.

Table 1
Clinical and sociodemographic characteristics of the studied participants.

Characteristics	RRP patients, n (%) (n = 14)	Controls, n (%) (n = 14)
Age, years (mean)	33.21	34.43
Gender		
Female	5 (35.7)	8 (57.1)
Male	9 (64.3)	6 (42.9)
Cigarette smoking status		
Current or former	3 (21.4)	0 (0)
Never	11 (78.6)	14 (100)
HPV type in biopsy specimens		NA
LR-HPV		
6	8 (57.1)	
11	4 (28.6)	
6/11	2 (14.3)	
HR-HPV		
16	0 (0)	
18	0 (0)	
SARS-CoV-2 infection ^a		
Yes	0 (0)	0 (0)
No	14 (100)	14 (100)

HPV: human papillomavirus; LR-HPV: low-risk HPV; HR-HPV: high-risk HPV; RRP: recurrent respiratory papillomatosis; NA: not applicable.

^a Prior to saliva collection.

3.3. HPV-Ct values and viral load dynamics in salivary cellular pellets

Ct values were assessed by qPCR assays to estimate differences in HPV viral load between saliva samples. In order to establish a cut-off by Ct values for HPV-positive and HPV-negative samples, serial 10-fold dilutions (100 to 0.1 ng; Pt. 02) were performed using PGMY09/11 primer set and resulted in Ct values of 18.87 (±0.07), 22.04 (±0.08), 25.57 (±0.12), and 29.82 (±0.18), respectively. Based on this, the cut-off for HPV-negative samples was Ct > 29.82 (±0.18).

As shown in Fig. 2, HPV-Ct values from salivary cellular pellets revealed that HPV-positive saliva of preoperative collections had a mean Ct = 24.33 (range: 22.96–25.85; ±1.25), whereas the HPV-negative saliva from patients in DFI had Ct ≥ 32.16. Fisher's exact test revealed significant difference in salivary HPV DNA detection between these two clinical moments (p = 0.021). Moreover, patients in DFI showed similar HPV-Ct values to healthy donors (Ct ≥ 34.18). Overall, salivary samples from all participants with HPV DNA detectable on gel electrophoresis exhibited a Ct ≤ 29.82 (±0.18), whereas the undetectable HPV DNA on gel showed a Ct > 29.82 (±0.18), as shown in our serial dilution curve.

Our next step was to investigate the HPV-Ct values in saliva among RRP samples using HPV-6 and HPV-11 primer sets. In HPV-positive saliva, the two patients carrying HPV-6 infection showed a mean HPV6-Ct = 26.80 (±0.16), whereas the three patients with HPV-11 infection had a mean HPV11-Ct = 23.86 (±2.66). For patients with HPV-negative saliva, HPV6-Ct values were ≥30.31 and HPV11-Ct values were ≥30.66.

By comparing paired samples (saliva-biopsy) from the 5 patients exhibiting HPV-positive saliva using PGMY09/11 primers, HPV-Ct values revealed a viral load difference of 5.13 Ct values between saliva and biopsies (mean Ct = 24.33 [±1.25] and 19.20 [±2.41], respectively). These findings may indicate the possibility of estimating the HPV viral load in the papillomatous lesions based on saliva samples.

A new saliva collection from one patient exhibiting HPV-positive saliva (Pt. 02; Ct = 23.70 [±0.01]) was performed two weeks after surgery. This new collection revealed undetectable salivary HPV DNA (Fig. 3).

3.4. CfDNA concentration and fragment size profile from saliva supernatant

Circulating cfDNA was obtained from saliva supernatant. The performance of cfDNA assays was compared between two commercial kits (Norgen and Qiagen). As shown in Fig. 4, Norgen and Qiagen yielded relatively similar salivary cfDNA concentrations between preoperative collections (range: 5.5–300 ng/mL and 5.31–207 ng/mL, respectively) and between healthy controls (range: 2.0–22.6 ng/mL and 1.32–25.2 ng/mL, respectively). For both kits, two patients (Pt. 03 and Pt. 06) exhibited higher salivary cfDNA levels compared to the other patients in the group. All other samples (patients and controls) exhibited cfDNA levels below 100 ng/mL. No significant difference was found between

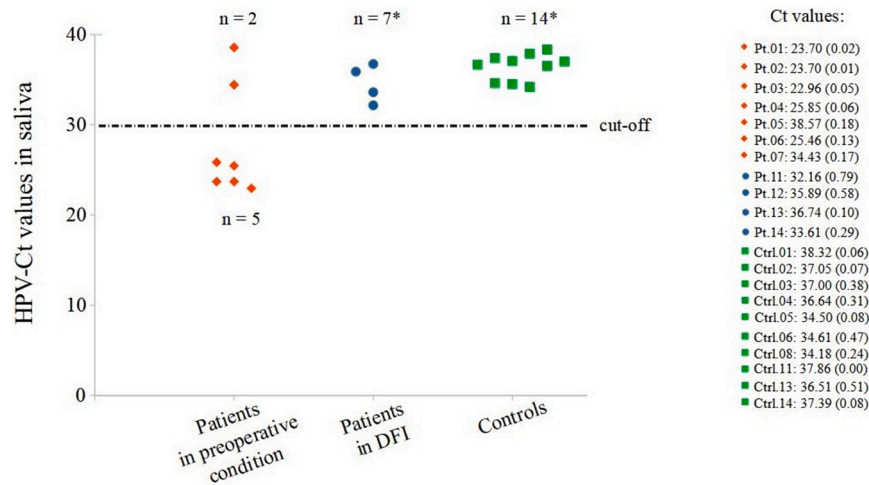


Fig. 2. HPV-Ct values from salivary cellular pellets of the study participants. HPV-Ct values using PGMY09/11 primers revealed HPV-positive saliva exhibiting a mean Ct = 24.33 (± 1.25) from samples of patients in preoperative condition (◆) compared with samples of patients in DFI (●) and healthy controls (■) (Ct ≥ 32.16 and Ct ≥ 34.18 , respectively). Asterisk (*) indicates the total number of participants in the group; those who are not shown in the figure (three patients in DFI and four healthy controls) had undetectable Ct values (Ct > 40). The horizontal line (—●—) represents the Ct value of 29.82 (± 0.18) as cut-off. Ct: Cycle threshold; DFI: Disease-free interval; Pt.: Patient; Ctrl.: Control.

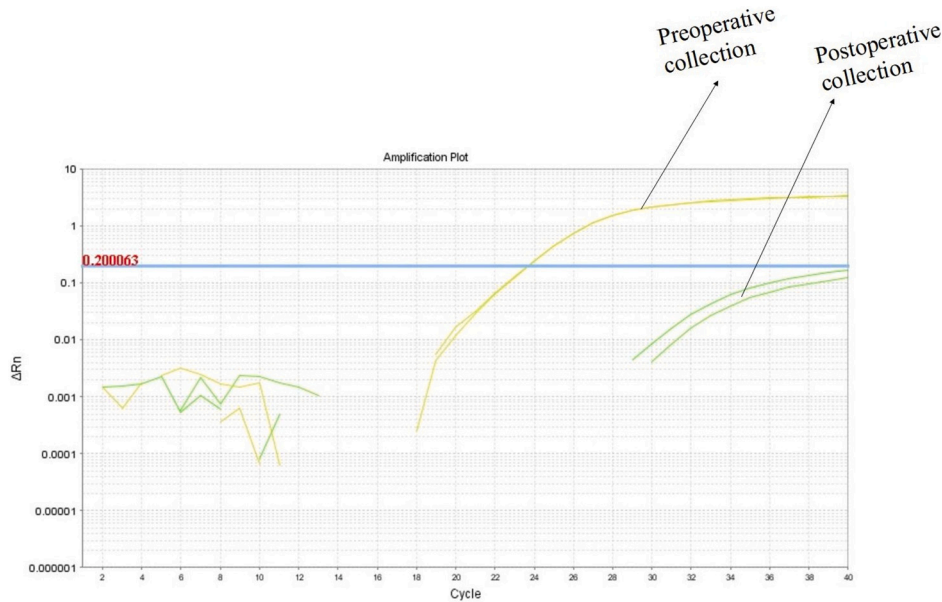


Fig. 3. HPV detection in saliva from Pt. 02. Differences observed in HPV-Ct values using PGMY09/11 detection for preoperative and postoperative saliva collections. Amplification plot curves: preoperative HPV-Ct = 23.70 (± 0.01); postoperative HPV-Ct = not detected (signal below the threshold line). Horizontal line: value of threshold line.

groups (Norgen: $p = 0.4697$; Qiagen: $p = 0.4615$).

Analysis of salivary cfDNA fragment size was obtained by Agilent TapeStation measurement. No evident peak of cfDNA fragment was detected in samples isolated from Norgen kit, whereas samples isolated from Qiagen kit resulted in cfDNA fragment size peaks identified in electropherograms. As shown in Fig. 5, cfDNA size distribution showed a variable profile between samples and a predominant size range of 150 to 400 bp was observed, corresponding to mononucleosomal (~ 170 bp) and dinucleosomal (~ 340 bp) fragments. Short cfDNA fragments (≤ 100 bp) and high molecular weight genomic DNA were also observed in some samples.

3.5. HPV cfDNA detection in saliva supernatant

Next, we investigated the circulating HPV cfDNA in saliva from all

preoperative collections of RRP patients by Ct values considering HPV-6 or HPV-11 detection according to HPV type in the papillomatous lesions. Salivary HPV cfDNA was detected in 42.9 % (3/7) of patients using Norgen kit and in 57.1 % (4/7) of patients using Qiagen kit. This means that, even exhibiting a lower viral load (Ct: 28.99 [± 0.14]), an additional patient with positive HPV cfDNA was detected using the Qiagen kit. Important to note, patients with HPV-positive saliva in cellular pellets but with HPV-negative cfDNA had insufficient amount of cfDNA input for qPCR performance.

3.6. Concordance of HPV DNA detection between papillomatous lesions and saliva

The concordance of HPV DNA detection between papillomatous lesions and saliva (cellular pellets and cfDNA) was assessed in all

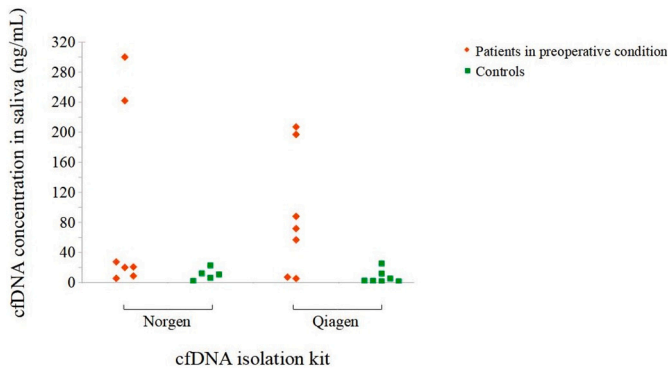


Fig. 4. Comparison of salivary cfDNA levels. cfDNA concentrations in saliva samples from patients in preoperative condition and healthy controls using Norgen and Qiagen kits and evaluated by Qubit® assay. Preoperative condition (n = 7); healthy controls (Norgen: n = 5; Qiagen: n = 7). Fisher's exact test revealed no significant difference between groups (p > 0.05).

preoperative collections using HPV-6 or HPV-11 primer set. Here, we are considering complete concordance as HPV DNA detection in papillomatous biopsy and saliva (cellular pellets plus cfDNA), partial concordance for papillomatous biopsy and saliva (cellular pellets or cfDNA), and no concordance for HPV DNA detection only in papillomatous biopsy. As shown in Table 2, complete concordance was observed in three patients (Pt. 02, Pt. 03, and Pt. 06) using Norgen kit and four patients

(Pt. 01, Pt. 02, Pt. 03, and Pt. 06) using Qiagen kit, partial concordance was found in one patient (Pt. 04) and no concordance was observed in two patients (Pt. 05 and Pt. 07).

4. Discussion

HPV DNA screening in saliva has been reported as a useful and non-invasive sampling source to identify individuals at risk of developing HPV-driven oral diseases, particularly oropharyngeal carcinoma. Additionally, the monitoring of HPV DNA in saliva from infected patients could complement the prognosis and treatment response. Weeramange et al. investigated patients with head and neck cancers (HNCs) using whole saliva and found HPV-positive saliva, especially HPV-16, in 43.2 % (212/491) of primary-HNC cohort and in 60 % (6/10) of those at recurrence [8]. Rettig et al. also reported common detection of HPV-16 DNA (67/124; 54 %) in oral rinse samples from patients with oropharyngeal carcinoma investigated at diagnosis, in which the HPV-16 DNA persistent detection (5 %) in posttreatment was associated with recurrent disease [21]. Tang et al. reported HPV-16 DNA detection in salivary oral rinse from a male with occult oropharyngeal cancer and undetectable viral load at 2 weeks post-tonsillectomy [7]. By investigating the cellular pellets from saliva, the present study only found HPV DNA from RRP patients in preoperative condition (5/7; 71.4 %), whereas patients in DFI and healthy donors showed undetectable HPV DNA in PCR-based assays. Similar to Tang et al. [7], we found undetectable HPV DNA in a new saliva collection at 2 weeks after surgery from a patient who

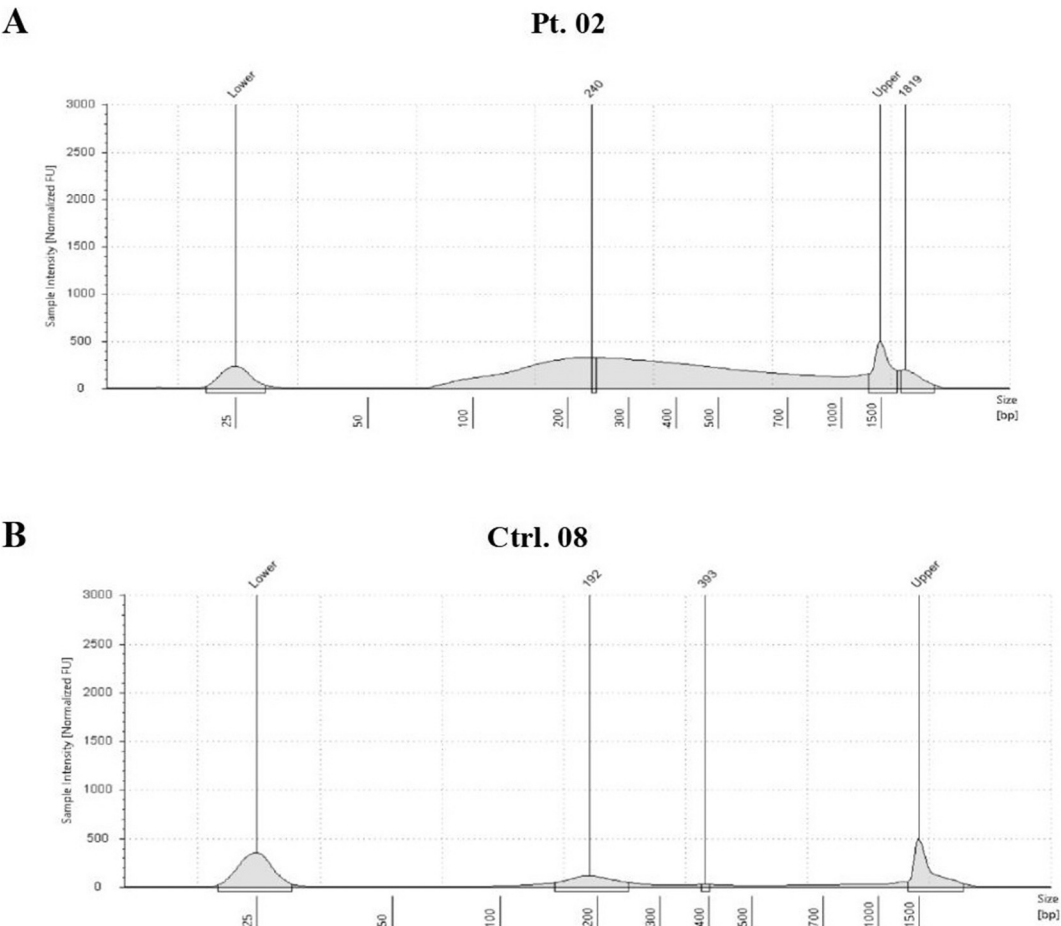


Fig. 5. Agilent TapeStation analysis for cfDNA in saliva. Representative electropherograms of cfDNA fragment sizes from saliva samples isolated with Qiagen kit. cfDNA size range of 150 to 400 bp was predominantly identified. Short cfDNA fragments (<100 bp) and high molecular weight genomic DNA can also be observed. (A) cfDNA lengths peaks in patient sample (Pt. 02: 240 bp and 1819 bp). (B) cfDNA lengths peaks in control sample (Ctrl.08: 192 and 393 bp). Pt.: Patient; Ctrl.: Control.

Table 2
PCR-based assays using HPV-6 or HPV-11 primer sets for HPV DNA detection in biopsy and saliva samples from patients undergoing surgery.

Patients	b_cPCR	b_qPCR; Ct values (SD)	s_cPCR	s_qPCR – Cellular pellets; Ct values (SD)	s_qPCR – cfDNA (by Norgen kit); Ct values (SD)	s_qPCR – cfDNA (by Qiagen kit); Ct values (SD)
Pt. 01	HPV6	22.96 (0.03)	Positive	26.69 (0.05)	Not detected	28.99 (0.14)
Pt. 02	HPV6	22.33 (0.02)	Positive	26.91 (0.01)	24.96 (0.03)	22.61 (0.04)
Pt. 03	HPV11	21.31 (0.26)	Positive	20.81 (0.05)	18.36 (0.02)	18.54 (0.54)
Pt. 04	HPV11	22.33 (0.01)	Positive	25.08 (0.04)	Not detected	32.79 (0.05) ^a
Pt. 05	HPV6	21.62 (0.07)	Negative	32.26 (0.33) ^a	Not detected	Not detected
Pt. 06	HPV11	16.07 (0.11)	Positive	25.70 (0.00)	23.83 (0.18)	15.98 (0.18)
Pt. 07	HPV6	18.72 (0.08)	Negative	30.31 (0.53) ^a	Not detected	35.34 (0.04) ^a

Pt.: patient; b: biopsy; s: saliva; cPCR: conventional PCR; qPCR: quantitative PCR; Ct: Cycle threshold; SD: standard deviation; not detected: signal below the threshold line.
^a HPV-Ct value considered as HPV-negative saliva.

exhibited HPV-positive saliva in the preoperative collection.

Regarding RRP patients, two studies reported HPV DNA screening from oral samples. Born et al. investigated 27 AoRRP and found HPV DNA in 25/27 of oral rinse samples and in 26/27 of oral swab specimens by conventional PCR assay using PGMY09/11 primers [22]. Hao et al. collected multiple oral rinses from 23 AoRRP every 90 days over 12 months and detected HPV DNA in 22 specimens by conventional PCR (PGMY09/11 primers), in which 77.2 % of them showed positive oral rinse in at least one of collections [23]. Unlike both previous RRP studies, our analysis involved saliva collection from RRP patients (9 AoRRP and 5 JoRRP) at two different clinical moments (preoperative and DFI) and could distinguished that HPV-positive saliva is only found in RRP patients with active disease. These findings suggest that active HPV replication in papillomatous lesions could shed viral DNA copies into saliva.

Our next step was to assess the HPV viral load in saliva through Ct values by qPCR assays. Ct values are inversely related to viral load dynamics and have been analyzed as indicator of infectivity and clinical progression in different viral pathologies like HPV and SARS-CoV-2 infections [15,16,24]. The Cobas® HPV test (Roche Molecular Systems) is an FDA-approved test to investigate 14 HR-HPV types in a single analysis. Duan et al. investigated cervical lesions using HPV-Ct values by Cobas® 4800 (HR) HPV test, in which a cut-point HPV-Ct < 31 was found for HPV positive women with sensitivity for CIN2+ and CIN3+ [15]. Inturrisi et al. evaluated cervical screening by comparing HPV testing on self-collected versus clinician-collected samples using Cobas® 4800 HPV test [16]. For self-collected samples, the mean Ct values of CIN3+, CIN2+, and <CIN2 were 31.54 (4.42), 31.33 (4.71), and 33.10 (4.99), respectively. For clinician-collected samples, the mean Ct values of CIN3+, CIN2+, and <CIN2 were 27.95 (3.93), 28.03 (4.20), and 31.85 (5.26), respectively. Additionally, the authors described a strong relation between CIN3+ risk and Ct value, being Ct value <30 similar for self-collected and clinician-collected samples. Regarding oral samples, Harlé et al. used Cobas® test to describe the HPV-Ct values in oral cytobrush samples from patients with HNCs [25]. The authors observed that the assay’s reproducibility provided a mean HPV16-Ct = 20.89 and a mean HPV18-Ct = 21.70 in oral cytobrush samples, whereas in FFPE samples a mean HPV16-Ct = 20.26 and a mean HPV18-Ct = 30.99 were found. In the present study, we evaluated the low-risk HPV (LR-HPV) in

saliva and biopsy specimens from RRP patients through Ct values using SYBR Green qPCR protocol. Based on serial dilutions, the cut-off Ct ≤ 29.82 (±0.18) using PGMY09/11 primers was found for HPV-positive saliva, in which preoperative collections, patients in DFI, and healthy donors exhibited Ct = 24.33 (±1.25), Ct ≥ 32.16, and Ct ≥ 34.18, respectively. To our knowledge, this is the first RRP study estimating salivary HPV viral load based on Ct values and distinguishing HPV-positive saliva during active disease.

Circulating cfDNA is the most commonly investigated category in liquid biopsy and some approved commercial tests based on cfDNA analysis are available for cancer investigations [26,27]. HPV cfDNA screening in biofluids could add information about disease status in HPV-related pathologies, being a useful diagnostic and prognostic biomarker. Hanna et al. showed that plasma HPV cfDNA levels from oropharyngeal cancer patients provided information about tumor burden, site of disease (regional or distant), and disease progression [13]. Important to note that even in HPV-related oral diseases, many of the liquid biopsy studies involving HPV cfDNA analysis are performed with plasma/serum samples. Here, salivary HPV cfDNA was detectable in 42.9 % (3/7) and 57.1 % (4/7) of RRP patients with active disease using the cfDNA kits from Norgen and Qiagen, respectively. Brooks et al. [28] investigated HPV cfDNA in saliva and plasma samples from three oropharyngeal carcinoma patients by droplet digital PCR and detected HPV16 DNA in 3/3 saliva samples and 2/3 plasma samples. The authors also described that the patient with HPV16 DNA detectable in saliva but not in plasma had earlier stage disease compared with the other two patients. Interestingly, Cui et al. [29] observed that cfDNA in saliva show greater sensitivity than cfDNA in plasma for oral tumors investigations.

The cfDNA fragment size is shorter than the cellular DNA and can be assessed by different methods, such as qPCR and gel electrophoresis [19]. In the present study, size profile of salivary cfDNA fragments was investigated by Agilent TapeStation system, an automated gel electrophoresis, and only saliva samples isolated from Qiagen kit resulted detectable cfDNA fragments. No prominent peak was observed in cfDNA samples isolated from Norgen kit. We observed that salivary cfDNA size range of 150 to 400 bp was predominantly identified in both patients and healthy controls. This profile is corresponding to mononucleosomal (~170 bp) and dinucleosomal (~340 bp) fragments [30,31]. Short cfDNA fragments (≤100 bp) and high molecular weight genomic DNA were also found in our analysis. Overall, these findings are similar to those described in literature, especially evaluating blood-derived cfDNA, the most studied biofluid in liquid biopsy. In contrast, information about the size profile of salivary cfDNA fragments is still limited in the literature. Brooks et al. [28] also found cfDNA fragments in saliva from a healthy donor of 100 to 300 bp range using the 2100 Bioanalyzer (Agilent Technologies). Based on qPCR of ALU fragments, Rapado-González et al. observed that longer salivary cfDNA fragments (≥247 bp) were significantly more prevalent in oral cancer patients compared with healthy controls [32]. In our study, no special difference was observed regarding the size of salivary cfDNA fragments between patients and controls, maybe due to the small cohort.

Currently, HPV testing on self-collected vaginal sample for the cervical cancer screening has been considered as accurate as a physician-collected sample and show high acceptability [33,34]. In the same way, the at-home exams with self-collection of saliva samples for HPV DNA screening combined with telehealth visit in otolaryngology should be considered to guide RRP evaluations, particularly for those patients with the most vulnerable socioeconomic status or located at long distances, since repeated visits to healthcare system to evaluate a possible recurrence can be discouraging. A study conducted by Ning et al. has shown that telemedicine in otolaryngology can offer user satisfaction and suitable images for diagnosis [35]. Therefore, the at-home exams could also increase screening for HPV-related oral diseases. Despite the high incidence rates of cervical cancer and oropharyngeal cancer in the Brazilian population, HPV detection and genotyping testing is not

commonly required in routine evaluations of public health systems in Brazil. PCR-based assays are sensitive and recommended for evaluations of HPV-related diseases [36]. Therefore, PCR-based assays should also be included as routine tests for HPV detection and genotyping in RRP evaluations. Since RRP can be misdiagnosed as asthma due to the similarity of clinical presentation, the findings of the present study reinforce that salivary HPV DNA could be a differential and non-invasive tool for monitoring the presence of papillomatous lesions in the upper airway during active disease.

Our pilot study has some limitations, including the small sample size, only two hospitals involved and located at the same city, the HPV detection assay only covering the most common types (HPV6, HPV11, HPV16, and HPV18), and the reduced amount of saliva supernatant (1 mL) used for cfDNA analyzes. However, this is the first RRP study to describe the HPV viral load in saliva through Ct values, including both salivary fractions (cellular pellets and cfDNA), and to distinguish that HPV-positive saliva is found in RRP patients with active disease. Together, all these findings reinforce that our study using SYBR Green qPCR protocol was suitable to describe how the manifestation and clinical course of RRP could be affected by the HPV viral load dynamics. Larger studies are needed to better elucidate the broad effects of HPV viral load on RRP clinical course.

5. Conclusions

Salivary HPV DNA can be an important non-invasive sampling source for clinical monitoring of infected individuals in the upper airway, such as RRP patients. HPV DNA in saliva can be assessed by both cellular pellets and circulating cfDNA. HPV-positive saliva may reveal active disease of RRP patients. Monitoring HPV viral load by Ct values is useful to complement the RRP clinical information.

CRedit authorship contribution statement

Mariana Chantre-Justino: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Marcelo Cardoso Figueiredo:** Conceptualization, Data curation, Resources, Investigation, Methodology, Writing – review & editing. **Gilda Alves:** Conceptualization, Data curation, Resources, Supervision, Writing – review & editing. **Maria Helena Faria Ornellas:** Conceptualization, Data curation, Funding acquisition, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

No conflict of interest.

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