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Comparative evaluation of the GP5 +/6 +, MY09/11 and PGMY09/11 primer sets for HPV detection by PCR in oral squamous cell carcinomas

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ABSTRACT

The aim of this study was to evaluate the use of GP5 +/6 +, MY09/11 and PGMY09/11 primer sets for the detection of human papillomavirus (HPV) DNA by single step polymerase chain reaction (PCR) and nested PCR in formalin-fixed and paraffin-embedded (FFPE) tissues from oral squamous cell carcinomas (OSCCs). DNA extracted from FFPE tissues were tested for amplification of the human beta globin gene with PCO3/4 primers. Positive samples for this gene were tested for HPV DNA using single step PCR with GP5 +/6 +, MY09/11 and PGMY09/11 primer sets. All negative samples at single step PCR with MY09/11 and PGMY09/11 were subjected to a further PCR with GP5 +/6 + primers using the non-amplified product in the previously reactions (nested PCR) as samples. Among 26 samples, 23 were positive for the human beta globin gene and were considered viable for HPV DNA detection by PCR. Single step PCR with GP5 +/6 + and MY09/11 primers and MY/GP + nested PCR did not amplify HPV DNA in any samples. PGMY09/11 primers detected HPV DNA in 13.0% of OSCC cases and this rate was raise to 17.4% with the use of PGMY/GP + nested PCR. According to our results the PGMY/GP + nested PCR is the most appropriate primer set for the detection of HPV DNA using FFPE samples from OSCC.

1. Introduction

Human papillomavirus (HPV) is a well-known causal agent of cervical and anogenital carcinomas (Zur Hausen, 2009). In addition, HPV has been considered an independent risk factor for development of a subset of head and neck squamous cell carcinomas (HNSCCs) (Smith et al., 2012). In the past few decades, an increase of HPV-positive cases in HNSCC has been reported (Chaturvedi et al., 2008; Näsman et al., 2009; Tinhofer et al., 2015). However, the association between HPV and oral squamous cell carcinomas (OSCCs) is still unclear and some results are contradictory. The reported prevalence of HPV DNA in oral cancer tissue has varied from 0 to 100% (Miller and Johnstone, 2001; Miller and White, 1996; Reyes et al., 2015; Rivero and Nunes, 2006).

According to the HPV status, OSCC has been divided into two classes, they have different molecular, clinical and pathological features. While HPV-negative HNSCC involves patients with long-term exposure to tobacco and/or alcohol, HPV-positive HNSCC develops independently of the use of these substances (Chaturvedi et al., 2008). Furthermore, HPV-positive tumors are more related to younger patients and sexual risk behavior, particularly with regard to the number of lifetime sexual partners and oral sex practice (Deschler et al., 2014; Gillison et al., 2008; Heck et al., 2010). Recent studies have shown that patients with HPV-positive HNSCC have higher response rates after treatment, improved overall survival and lower risks of progression and death than those with HPV-negative HNSCC (Ang et al., 2010; Fakhry et al., 2008; Young et al., 2015). Therefore, it is supposed that it may be a reduction of therapy intensity, in order to reduce treatment-related toxicity (Panwar et al., 2014) and it is conceivable that treatment strategies may target specific molecular pathways that differ between HPV-positive and HPV-negative HNSCC (Venuti and Paolini, 2012).

Thus, the assessment of HPV status in HNSCC has important clinical relevance, yet no consensus on the most reliable method for this analysis has been established. The standard protocol should be highly accurate, technically feasible, cost effective, and readily transferrable to diagnostic pathology laboratory (Westra, 2014).

Currently, the molecular detection of HPV DNA is the gold standard for virus detection in tissue samples and exfoliated cells (Zaravinos et al., 2009). PCR is a highly sensitive technique, which can be used for a wide variety of samples, including formalin-fixed, paraffin-embedded (FFPE) tissues (Venuti and Paolini, 2012; Westra, 2014). The aim of this study was to evaluate the use of GP5 +/6 +, MY09/11

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and PGMY09/11 primer sets for the detection of HPV DNA by single step polymerase chain reaction (PCR) and nested PCR in FFPE oral tissues from patients with OSCC.

2. Material and methods

2.1. Study samples

This study was approved by the Ethics Committee in Human Research of the authors' institution (approval number 144.430). Cases were selected from histopathological reports of the Oral Pathology Laboratory, Federal University of Santa Catarina (Florianópolis, Brazil). Clinical data were collected from biopsy reports. A total of 26 cases of OSCC were included in this study. Sections of 10 μm were obtained from FFPE tissues to perform the DNA extraction.

2.2. DNA extraction

Samples were subjected to deparaffinization with pre-heated xylene and DNA extraction was performed as previously described (Rivero et al., 2006; Rivero and Nunes, 2006). DNA concentration and purity were evaluated by Nanovue™ Plus Spectrophotometer (GE Healthcare). Samples had a purity ratio (260/280 nm absorbance) between 1.5–1.9 and were diluted in purified water to near 100 ng/μl prior to PCR.

2.3. PCR analysis

The PCRs were conducted using the Mastercycle Personal® (Eppendorf, Germany) and the primer sets are listed in Table 1 in accordance with the algorithm described in Fig. 1.

DNA integrity and absence of PCR inhibitors were tested by amplification of the human beta globin gene with PCO3/4 primers (Saiki et al., 1988). For this, 4 μl of DNA was amplified in a total volume of 20 μl, with a final concentration of 1.5 U de Taq Platinum DNA polymerase (Invitrogen™ Life Technologies), 1× PCR buffer (Tris–HCL 200 mm, pH 8.4; KCl 500 mm), 0.25 mm of dNTP (dATP, dTTP, dCTP, dGTP), 1.5 mm of $MgCl_2$, 12 μg/ml of bovine serum albumin (BSA) and 10 ρmol of each primer. The PCR cycling conditions were 40 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min and a final extension at 72 °C for 7 min. The PCR products were electrophoresed using a 2.5% agarose gel in 1× TAE buffer, stained with ethidium bromide (0.5 g/ml) and photographed under UV-illumination. A low DNA mass ladder was used as a base-pair molecular weight pattern (DNA ladder 100 pb, Invitrogen ™ Life Technologies).

A single step PCR assay using the GP5+/6+ consensus primers (de Roda Husman et al., 1995) was performed to amplify a sequence of 150 pb from the L1 region of HPV. The amplification was carried out in the same way described above with 1 U de Taq Platinum DNA polymerase (Invitrogen™ Life Technologies), 0.5 μM of each primer and 5 μl of DNA. The PCR cycling conditions were 40 cycles of 94 °C for 1 min, 40 °C for 1 min, 72 °C for 1 min and a final extension at 72 °C for 5 min.

Similarly, a single step PCR using the MY09/11 primers (Şahiner et al., 2014) was performed to amplify a sequence of 450 pb from the L1 region of HPV. The amplification was carried with 5 U de Taq Platinum DNA polymerase (Invitrogen™ Life Technologies), 10 ρmol of each primer and 5 μl of DNA. The PCR cycling conditions were 40 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min and a final extension at 72 °C for 5 min.

The samples were also screened for the presence of HPV using PGMY09/11 (Gravitt et al., 2000) primers, which also amplify a sequence of 450 pb from the L1 region of HPV (Fuessel Haws et al., 2004). The amplification and the PCR cycling conditions were the same that described for MY09/11 primers.

The electrophoresis for all three PCR products of amplification (GP5+/6+, MY09/11 and PGMY09/11) was similar to electrophoresis for products of amplification of the human beta globin gene, with the exception of the 2% agarose gel. All PCR assays were performed using purified water in place of DNA sample as a negative control. For positive control, it was used a FFPE cervical tissue from a cervical intra-epithelial neoplasia HPV-positive case, confirmed by histopathological analysis, which was subjected to same deparaffinization and DNA extraction as other samples.

All negative samples at single step PCR using MY09/11 and PGMY09/11 primer sets were subjected to a further PCR with GP5+/6+ primers as described before and using as samples 1 μl of non-amplified product in previously reactions (nested PCR).

Table 1
Sequences of primers used in PCRs.

Primers	Sequence (5′–3′)
PCO3	ACA CAA CTG TGT TCA CTA GC
PCO4	CAA CTT CAT CCA CGT TCA CC
GP5+	TTT GTT ACT GTG GTA GAT AC TAC
GP6+	GAA AAA TAA ACT GTA AAT CA TAT TC
MY09	CGT CCM ARR GGA WAC TGA TC
MY11	GCM CAG GGW CAT AAY AAT GG
PGMY11-A	GCA CAG GGA CAT AAC AAT GG
PGMY11-B	GCG CAG GGC CAC AAT AAT GG
PGMY11-C	GCA CAG GGA CAT AAT AAT GG
PGMY11-D	GCC CAG GGC CAC AAC AAT GG
PGMY11-E	GCT CAG GGT TTA AAC AAT GG
PGMY09-F	CGT CCC AAA GGA AAC TGA TC
PGMY09-G	CGA CCT AAA GGA AAC TGA TC
PGMY09-H	CGT CCA AAA GGA AAC TGA TC
PGMY09-Ia	G CCA AGG GGA AAC TGA TC
PGMY09-J	CGT CCC AAA GGA TAC TGA TC
PGMY09-K	CGT CCA AGG GGA TAC TGA TC
PGMY09-L	CGA CCT AAA GGG AAT TGA TC
PGMY09-M	CGA CCT AGT GGA AAT TGA TC
PGMY09-N	CGA CCA AGG GGA TAT TGA TC
PGMY09-Pa	G CCC AAC GGA AAC TGA TC
PGMY09-Q	CGA CCC AAG GGA AAC TGG TC
PGMY09-R	CGT CCT AAA GGA AAC TGG TC
HMB01b	GCG ACC CAA TGC AAA TTG GT

Set of primers used and their sequences: PCO3/4, GP5+/6+ and MY09/11 consist of one forward and one reverse primers; PGMY09/11 comprises five forward primers (11-A to 11-E) and 13 reverse primers (09-F to HMB01). The degenerate bases code to MY09 and MY11 is: M = A or C, W = A or T, Y = C or T or R = A or G.

2.4. Statistical analysis

The level of agreement between the methods was indicated by Kappa values of 0–0.2 (poor), 0.21–0.4 (fair), 0.41–0.6 (moderate), 0.61–0.8 (good) and 0.81–1.0 (very good). Statistical analyses were performed with MedCalc software (version 14.8.1).

3. Results

Patients were mostly men (91.3%) (21/23) and only 8.7% (2/23) were women. The median age was 61 years old, ranging from 41 to 80 years. Tumor's primary sites included floor of mouth (7/23), soft and/or hard palate (6/23), tongue (4/23), retromolar trigone (3/23) and jugal mucosa (3/23). Furthermore, 73.9% (17/23) of the patients reported tobacco use and 52.2% (12/23) reported both tobacco use and alcohol consumption.

The single step PCR GP5+/6+ and MY09/11 did not amplify the HPV DNA in any samples, while single step PGMY09/11 detected HPV DNA in 13.0% (3/23) of the OSCC cases. HPV amplification was also not observed in any case by nested MY/GP+. On the other hand, one more sample was positive for the virus with the use of nested PGMY/GP+. At the end, 17.4% (4/23) were HPV-positive OSCC.

Statistical analysis indicated no agreement with the comparison of those three single step PCR methods each other (all kappa values

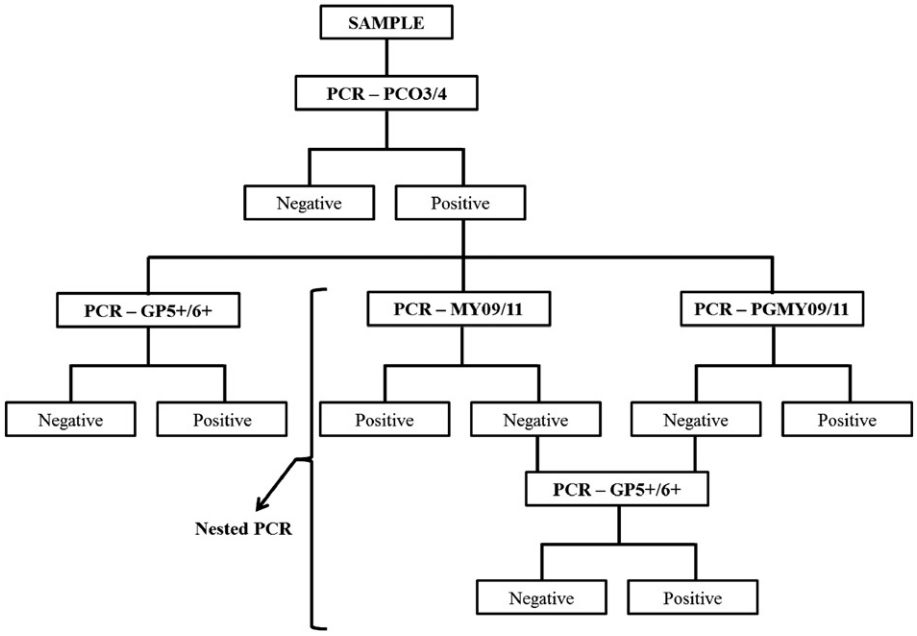

Fig. 1. Algorithm of the performed PCRs: Samples of a total of 26 FFPE oral tissues from patients with OSCC were tested with PCO3/4 primers for amplification of the human beta globin gene. Positive samples in this reaction were tested for HPV DNA using single step PCR with GP5+/6+, MY09/11 and PGMY09/11 primer sets. All negative samples at single step PCR with MY09/11 and PGMY09/11 were subjected to a further PCR with GP5+/6+ primers using as samples 1 μl of the no amplified product in the previously reactions (nested PCR).

equal 0.0; 95%, CI = 0.0). No agreement (k = 0.0; 95%, CI = 0.0) was also observed when two nested PCR methods were compared.

4. Discussion

Due to the distinctive character of HPV-positive HNSCC, HPV detection as part of the routine diagnosis has been widely discussed (Westra, 2014). The standard protocol for detection of HPV have not yet been defined, which has generated doubts about when and why to assess HPV status. Thus, HPV test is not requested or is ordered indiscriminately, without any contextual relationship with anatomic site, microscopic findings, clinical relevance, or other factors, which may influence the importance of virus detection (Westra, 2014).

In this study, PCR with PCO3/4 primers for amplification of human beta globin gene revealed that protocols of paraffin removal and DNA extraction for FFPE oral tissue samples, that were used, allowed to obtain 88.5% (23/26) of feasible samples for PCR analysis. In clinical practice this would mean that 11.5% (3/26) of patients would not have the possibility of assessing the status of HPV from those samples by PCR.

Others studies have shown that DNA in FFPE tissue specimens could be degraded to short fragments (smaller than 250 base pairs), because of fixative-induced cross-links. Therefore, only PCR primer sets, such as GP5+/6+, which generates short PCR products, could obtain reliable results for those specimens (Snijders et al., 2010). In our study, GP5+/6+ primers were not able to amplify the HPV DNA in any samples. Another study performed in Brazil by Rivero and Nunes (2006) using the single step PCR with GP5+/6+ primers, analyzed 23 FFPE and 17 fresh tissues from OSCC and the detection rate for HPV DNA was also 0.0% (Rivero and Nunes, 2006).

In the present study, the MY09/11 and nested PCR MY/GP+ assays did not amplify the HPV DNA for any samples. Although, another study carried out in Brazil by Miguel et al. (1998), tested 45 frozen HNSCC samples and detected 11% of HPV-positive tumors using MY09/11 primer set. Miyahara et al. (2011) analyzed 83 FFPE tissues from patients with SCC by nested PCR MY/GP+ and detected 33.7% HPV-positive tumors.

The present study revealed that PGMY09/11 was the only primer set able to detect the HPV DNA in our samples. Our results were improved by subsequent PCR GP5+/6+ (nested PCR PGMY/GP+). This finding is consistent with previous studies showing that nested PCR may be required to confirm negativity or to detect low levels of HPV (Winder et al., 2009).

Although, in our study no agreement was observed when compare GP5+/6+, MY09/11 and PGMY09/11 primer sets at a single step PCR, Winder et al. (2009) found a moderate agreement between those three PCR methods by testing 34 clinically diagnosed samples of genital warts, cervical and vulval biopsies. Furthermore, Fuessel-Haws et al. (2004) analyzed 37 cervical smear samples from women with cervical dysplasia or a history of cervical dysplasia. They obtained a very good agreement between the first PCR step MY09/11 versus PGMY09/11, and also between nested PCR MY/GP+ versus PGMY/GP+, even though the PGMY/GP+ has detected more HPV types than the MY/GP+.

PGMY09/11 primers each consists of oligonucleotide pools that bind to the same region as the MY09/11; but they are not degenerate primers, which provide a measure of quality assurance in primer design (Fuessel Haws et al., 2004). According to Gravitt et al. (2000), PGMY09/11 primers demonstrated higher sensitivity and ability to detect a wider variety of HPV types, especially in cases of multiple infections. They also detected HPV 26, 35, 42, 45, 52, 54, 55, 59, 66, 73 with at least 25% more frequently than MY09/11 primers (Gravitt et al., 2000).

Several methods currently available are able to provide information about the presence of HPV in biological samples and each have its advantages and disadvantages. For most clinical laboratories, combination of a sensitive test, such as PCR, and a specific test allows the best potential to determine the HPV status in a given sample. However, choosing the most suitable method must be made from practical considerations, as well as the intention of its use (Venuti and Paolini, 2012).

In summary, the detection rate of HPV-positive OSCC was 17.4%. PGMY/GP+ nested PCR was the most appropriate primer set for the detection of HPV DNA in FFPE samples from OSCC. Further studies are necessary to identify the most reliable method and to determine the standard protocol for the detection of HPV DNA in OSCC.

Conflict of interest

The authors declare no conflict of interest.

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