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HPV detection in oral mucosa samples in pediatric patients

Detecção de HPV em amostras de mucosa oral em pacientes pediátricos

Aline R. Gama; Marcos Antonio B. Carvalho Jr.; Isabela J. Wastowski; Stela O. Rodrigues; Maria Fernanda B. Souza; Lais S. Botacin; Melissa A. G. Avelino; Lilian Carla Carneiro

Universidade Federal de Goiás, Goiânia, Goiás, Brazil.

ABSTRACT

Introduction: The human papillomavirus (HPV) detection favors treatments for patients with clinical manifestations and limits future consequences for those with asymptomatic infections. **Objectives:** Therefore, the present study aimed to evaluate the sensitivity of polymerase chain reaction (PCR) for HPV detection from oral mucosa samples, of asymptomatic patients and patients with clinical manifestations of laryngeal papillomatosis. **Material and methods:** A total of 49 pediatric patient samples were obtained by exfoliation of the oral mucosa with a sterile brush. The deoxyribonucleic acid (DNA) samples was extracted and used for HPV detection, using GP5 and GP6 oligonucleotides, by conventional PCR and qPCR reactions. **Results:** Among the 49 samples, eight were from patients clinically diagnosed with laryngeal papillomatosis, but in both conventional PCR and qPCR technic, only one sample had presented positivity. **Discussion:** These results suggest that the sample type, the methodology used to collect, the extraction methodology used, the anatomical location of the lesion and the oligonucleotides used; all factors strongly influence the sensitivity of HPV detection by PCR methodology. **Conclusion:** Thus, more studies are needed to better determine the sample collection, and the processing techniques present more reproducibility on PCR detection.

Key words: papilloma; polymerase chain reaction; DNA primers; biopsy.

RESUMO

Introdução: *A detecção do papilomavírus humano (HPV) auxilia os tratamentos para pacientes que apresentam manifestações clínicas e limita as consequências futuras para os que apresentam infecções assintomáticas.* ***Objetivos:*** *Avaliar a sensibilidade da reação em cadeia da polimerase (PCR) para detecção de HPV em diferentes amostras.* ***Material e métodos:*** *Quarenta e nove amostras de pacientes pediátricos foram obtidas por esfoliação da mucosa oral com uma escova estéril. O ácido desoxirribonucleico (DNA) dessas amostras foi utilizado para detecção de HPV por PCR convencional e PCR em tempo real (qPCR).* ***Resultados:*** *Das 49 amostras, oito eram de pacientes clinicamente diagnosticados com papilomatose laríngea; porém, tanto na PCR convencional quanto na qPCR, apenas uma amostra apresentou amplificação do fragmento esperado.* ***Discussão:*** *Esses resultados sugerem que o tipo de amostra, a metodologia empregada na coleta, a metodologia de extração empregada, a localização anatômica da lesão e os oligonucleotídeos utilizados influenciam fortemente a sensibilidade da detecção de HPV por PCR.* ***Conclusão:*** *Mais estudos são necessários para determinar as melhores técnicas de coleta e processamento das amostras a fim de que a detecção de HPV por PCR seja mais eficiente.*

Unitermos: *papiloma; reação em cadeia da polimerase;* primers *do DNA; biópsia.*

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RESUMEN

Introducción: *El virus del papiloma humano (VPH) ayuda los tratamientos de pacientes que presentan manifestaciones clínicas y limita las consecuencias futuras para aquellos con infecciones asintomáticas.* ***Objetivos:*** *Evaluar la sensibilidad de la reacción en cadena de la polimerasa (PCR) para detectar VPH en diferentes muestras.* ***Material y métodos:*** *Cuarenta y nueve muestras de pacientes pediátricos se obtuvieron por exfoliación de la mucosa oral con un cepillo estéril. Se utilizó el ácido desoxirribonucleico (ADN) de esas muestras para detectar VPH por PCR convencional y PCR cuantitativa en tiempo real (qPCR).* ***Resultados:*** *Entre las 49 muestras, ocho eran de pacientes clínicamente diagnosticados con papilomatosis laríngea; sin embargo, tanto en la PCR convencional como en la qPCR, sólo una muestra presentó amplificación del fragmento esperado.* ***Discusión:*** *Eses resultados sugieren que el tipo de muestra, el método empleado en la recolección, el método de extracción, la ubicación anatómica de la lesión y los oligonucleótidos utilizados influyen fuertemente la sensibilidad de detección de VPH por PCR.* ***Conclusión:*** *Se necesita mayor investigación para determinar las mejores técnicas de recolección y procesamiento de muestras para que la detección de VPH por PCR sea más eficiente.*

Palabras clave: *papiloma; reacción en cadena de la polimerasa; cartilla de ADN; biopsia.*

INTRODUCTION

Characterized as a deoxyribonucleic acid (DNA) virus, the human papillomavirus (HPV) can cause the development of different types of lesions that affect the skin and mucous membranes. About 13 types of HPV, among the more than 150 already described, are associated with the occurrence of several cancers, among which those affecting the gynecological and cervical regions stand out[1].

Laryngeal papillomatosis is also one of the presentations of HPV infection, which can manifest in both children and adults, being more prevalent in the first group. Its incidence is estimated at 4.3 cases per 100 thousand children in the United States[2]. The disease may present in several forms, being classified as aggressive (need for 10 or more surgical procedures, three or more within a year, or distal extension to the subglottis) and non-aggressive (less than 10 procedures required, with less than three procedures being performed within one year, and no distal involvement)[3].

The most common manifestations are those with warty or papillomatous lesions with progressive growth, which may lead to the imminent risk of airway obstruction. They are usually caused by HPV types 6 and 11. In some patients, spontaneous remission of lesions may occur. In other cases, lesions may undergo malignant transformation (1%-4%)[4].

HPV route of transmission is not yet fully understood, but sexual and non-sexual transmissions are recognized, including the vertical form, especially during childbirth. Contamination can occur even in children of mothers with no previous or current history of genital papillomas[5].

Although the clinical implications of HPV infection in children are known, there are few epidemiological studies on the prevalence of viral type in the pediatric population. Such studies are extremely relevant, since there is a high incidence of HPV in the sexually active population, and consequently, strong possibility of estimated vertical transmission in 50%-83.3% of infected mothers. Although there is a large number of infected children, only about 2% of them develop laryngeal papillomatosis or other clinical implication, leading to the understanding that there is a huge amount of asymptomatic pediatric patients[6].

Given the growing relationship between HPV and the pediatric population, the detection of this virus increases the effectiveness of treatments for patients with clinical manifestations and limits future consequences for those with asymptomatic infections, increasing both survival and quality of life for these patients[7]. Thus, the establishment of accessible and reliable HPV collection and tracking methodologies is important. In this scenario, the polymerase chain reaction (PCR) stands out, which is considered as the most sensitive technique for detection of viral DNA, and is therefore an excellent method for identification of HPV in human tissues[8].

Although PCR is a highly efficient technique for HPV detection, the success of this procedure depends on the lesion site, the presence or absence of keratinization, and the method used to collect the sample. One of the most common used methods is the biopsy, because this material enables the recovery of cells present in the basal layer, where HPV is usually in its latent form. However, performing the biopsy is dependent on the presence of a physician and proper surgical instruments, making this method relatively expensive and unavailable in some health care units.

In this situation, the use of sample collection brushes and swabs makes the removal of mucosal cells more accessible and a good alternative for obtaining high DNA concentration without invasive procedures[8, 9].

OBJECTIVES

This study aimed to evaluate the sensitivity of PCR for HPV detection in oral mucosa samples from asymptomatic pediatric patients and patients with laryngeal papillomatosis.

MATERIAL AND METHODS

Patients

A molecular study was carried out with 49 samples obtained from children, aged 12 years and under, who were undergoing treatment and follow-up at the otorhinolaryngology outpatient clinic at Universidade Federal de Goiás (UFG), Hospital das Clínicas, Goiânia city, Goiás state, Brazil. Two groups of individuals were evaluated: patients who had clinically diagnosed laryngeal papillomatosis and patients with other clinical conditions not associated with HPV infection.

Inclusion criterion in this study was: being up to 12 years of age at the time of collection. Exclusion criteria were: patients immunized against HPV, patients older than 12 years at the time of collection. The study protocol was approved by the Research Ethics Committee of Hospital das Clínicas of the UFG, under protocol number 016/2017. The informed consent form was obtained from all people responsible for the patients under study.

Sample collection and processing

For HPV molecular detection, the samples were collected by exfoliating the oral mucosa, using a sterile brush. In one of the patients, the sample was collected directly by exfoliating the lesion. After collection, the samples were stored at approximately 4°C for a maximum of three days until processing.

Molecular detection

DNA from the sample brushes was extracted using the PureLink Genomic DNA extraction kit (Invitrogen, Carlsbad, California, USA), according to the manufacturer's recommendations. After extraction, the DNA was stored at approximately -20°C until processing.

HPV sequence detection was performed by conventional PCR and real-time quantitative PCR (qPCR) using GP5+ (5'-TTTGTTACTGTGGTAGATACYAC-3) and GP6+ (5'-GAAAAATAAACTGTAAATCATATTC-3') primers, amplifying a 150 bp fragment of the highly conserved L1 HPV gene[10]. Conventional PCR reactions were performed in a thermal cycler (MJ Research, Inc., Quebec, Canada), according to standardized methodology by Venceslau *et al*. (2014)[11], with some modifications. The reaction contained 1 µl deoxyribonucleotide triphosphate (DNTP) (20 µM); 0.4 µl HotStart Taq DNA Polymerase (5 U); 5 µl MgCl (50 mM); 2 µl each primer (10 mM); 10 µl each sample and ultrapure water (Millipore) to complete a final volume of 50 µl. The conditions for this PCR were: initial denaturation for one minute at 95°C, followed by 30 cycles of denaturation for 30 seconds at 95°C, annealing for 30 seconds at 48°C and extension for three minutes at 72°C and final extension for two minutes at 72°C. The amplicons were analyzed by 2% agarose gel electrophoresis stained with blue green loading dye (LGC Biotecnologia, São Paulo, Brazil) and photographed using a photo documentation system.

The qPCR reactions were performed at a thermocycler iQ5 (Bio-Rad, Hercules, California, USA), using 4 µl Hot FirePol (5 X); 0.4 µl each primer (10 pMol); 1 µl each sample (4 ng/µl) and ultrapure water to complete a final volume of 20 µl. The conditions for this qPCR were: initial activation for 12 minutes at 95°C, followed by 40 cycles of denaturation for 15 seconds at 95°C, annealing for 30 seconds at 60°C and extension for 30 seconds at 72°C. At the end of the cycles, the samples were subjected to melting analysis at 65°C-95°C. After completion of the reaction, the generated files were visualized in the equipment software. Samples with melting temperature (Tm) close to 80°C were considered positive[12].

RESULTS

Among the 49 samples, 41 were from asymptomatic patients, and eight samples were from patients with a clinical diagnosis of laryngeal papillomatosis. HPV DNA was detected in only one sample out of eight that were extracted from patients known to have a lesion. This detection was performed by both conventional PCR and qPCR. The positive sample was the only one among the others that was collected with exfoliation of the lesion itself caused by laryngeal papillomatosis, thus suggesting that the presence of the lesion and the collection procedure are crucial for the detection of HPV DNA by PCR. **Figure 1A** shows the agarose gel electrophoresis where we can observe the presence of a 150-bp fragment corresponding to the expected amplification.

The qPCR reaction, performed with the same samples as conventional PCR, generated a melting curve presenting a single positive sample with Tm equal to 77°C, corresponding to the specific Tm for this amplification (**Figure 1B**).

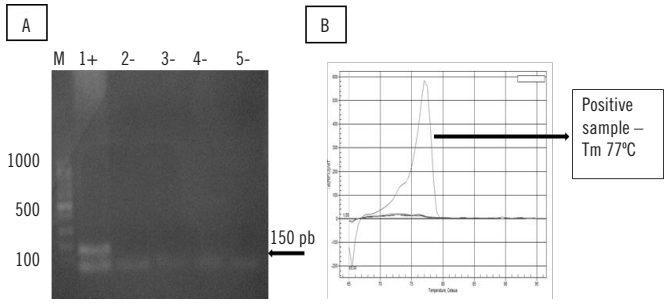

FIGURE 1 – *A) agarose gel electrophoresis; B) HPV melting curve obtained by amplifying the L1 region flanked by GP 5+ and GP 6+ primers*

M: molecular weight marker; 1: positive sample; 2 to 5: negative samples; HPV: human papillomavirus.

DISCUSSION

The method employed in the collection of material for HPV DNA detection by PCR is decisive for the effectiveness of this procedure. This fact was observed in our study, since it was only possible to detect HPV DNA in the sample collected directly from the lesion. Castro and Filho[13] observe that even using sensitive techniques such as PCR, the detection rate may vary from 0% to 100%, and mention that the large difference between the results can be attributed to errors at the time of collection and highlight the great difficulty in performing detection HPV in swabs and biopsy samples.

Different rates of HPV DNA detection by PCR are observed in recent studies. This difference suggests a variation in the ability to amplify DNA fragments of different sizes and specific HPV types, and this difference may also be linked with the types of materials used (smears, frozen material, or paraffin-embedded), the anatomical location of the injury, population issues and oligonucleotide designs[11].

Tristão *et al*. (2012)[14] performed conventional HPV PCR detection on 125 samples of oral mucosa scrapings collected using an endocervical brush, and viral DNA was found in only 29 samples (23.2%). According to the authors, the samples were extracted from randomly chosen men and women.

In another study, researchers found a percentage of positive samples similar to the above, but for DNA extraction they did not use swabs or brushes, they used tumors in paraffin block, collected from patients with histological diagnosis of oral cavity and oropharynx squamous cell carcinoma. Of the 82 tumors extracted, 21 (25.6%) had HPV DNA[15]. This result suggests that the rate of viral DNA obtained from cell collection with brushes or swabs is very close to that obtained by direct extraction of biopsy samples[9].

Obtaining only one positive sample in our work may still be justified by the use of a commercial viral DNA extraction kit. Abrão *et al*. (2005)[9] performed a comparison between DNA extraction from oral mucosa cells, using NaCl and commercial kit extraction, and observed that the DNA obtained by extraction with the kit was of better quality, but had lower concentration, and larger volumes were needed for PCR reactions performed. According to the authors, commercial kit extraction presents higher cost, lower yield and longer execution time when compared to extraction with NaCl. The use of DNA extraction from mucosal cells, using NaCl, could be an option to increase the DNA concentration obtained in the samples of this study, thus enabling detection of viral DNA.

CONCLUSION

The present study shows that the use of conventional PCR and qPCR for HPV detection in samples of asymptomatic pediatric patients, with clinically evaluated laryngeal papillomatosis, had low sensitivity. The sample type collected or even the DNA extraction kit used may have potential interference in the obtained results. Further studies are needed to determine a DNA collection and extraction technique to make PCR detection more efficient.

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CORRESPONDING AUTHOR

Aline Rodrigues Gama ID 0000-0003-2167-3872
e-mail: alinerodriguesgama15@gmail.com