Comparison between Two Methods for Molecular Characterization of Human Papillomavirus

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ABSTRACT

Introduction: a sensitive method of detection for Human Papillomavirus (HPV) is important to facilitate the early treatment of cervical cancer precursors. **Objective:** to analyze the spectrum of HPV infection and compare the sensibility of DNA HPV detection using polymerase chain reaction (PCR) and nested PCR (nPCR) methods in a group of 251 women of Pelotas-RS. **Methods:** genomic DNA was extracted from the collected samples and was submitted to PCR methods with the primers MY09/11 and nPCR with the pair of primers MY09/MY11 and GP5+/6+. The results were applied to the softwares Epi-Info v.3.5.1® and STATA v.11® for analyzes. **Results:** the prevalence of HPV infection was 6.8% with the use of primers MY09/11. When associated with primers GP5/6, this result increased to 29.9% (p < 0.001). **Conclusion:** the increase founded in HPV DNA detection from 6.8 to 29.9% suggests that the technique of nPCR MY09/11 followed by GP5/6 is the most sensitive method to detect HPV DNA from cervical specimens.

Keywords: HPV, molecular diagnostics, nested PCR

INTRODUCTION

Estimates show that about 291 million women worldwide are infected with human papilloma virus (HPV)[1]. According with the Brazilian Ministry of Health, Brazil is one of the world leaders in the incidence of HPV, with 137,000 new cases of infection each year[2]. With the introduction of biomolecular techniques, it was possible to confirm that the cervical cancer (CC) development is closely associated with the HPV, showing the significance of the HPV infection for the dysplasia development and the transformation of normal cervical cells into cancerous[3,4]. This type of cancer is the responsible for the death of 31.400 women in Latin America, 11.000 only in Brazil[5,6] each year. Even though HPV infection can be detected by preventive examinations for CC (Pap test), or pathological (by colposcopic directed cervical biopsy), this is often not possible. However, the presence of HPV can be confirmed with high accuracy, by identifying the viral genome present in cervical lesions[7].

There are currently several techniques for the molecular diagnosis of HPV, ranging from a conventional Polymerase Chain Reaction (PCR) methods complex, such as real-time PCR, hybrid capture (HC) and microarray[8,9]. The PCR technique is still considered the "gold standard" for HPV diagnosis, as the DNA-target is selectively amplified. However, this characteristic makes the method susceptible to contamination by exogenous or amplified nucleic material from another sample[10]. A variation of this technique called nested-PCR (nPCR) with the MY09/11 and GP5+/6+ primer sets, is a high sensitive specific method for HPV DNA detection[11], whith both targeting the L1 conserved region of the viral genome, allowing the detection of a broad range of HPV types[12].

The widely used MY09/11 consensus primers set is synthesized with several degenerated nucleotides in each primer and is thus a mixture of 25 primers, targets a 450bp conserved sequence in the HPV L1 gene, and is therefore able to amplify a broad spectrum of HPV types[13]. The GP5 and GP6 primer set consists of a fixed nucleotide sequence for each primer and detects a wide range of HPV types by using a lowered annealing temperature during PCR, and targets a 140bp sequence of HPV L1 gene, located inside the sequence recognized by the MY primers[12].

OBJECTIVE

Considering that HPV infection is an integral part of CC development, the viral genome detection can be used as a surveillance strategy, to identify HPV infected women and to monitor the progression of cervical lesions, as it is a disease of high mortality that can be prevented[7]. Based on these data, this study aimed to analyze the spectrum of HPV infection and compare the sensibility of DNA HPV detection using polymerase chain reaction (PCR) and nested PCR (nPCR) methods in a group of 251 women of Pelotas-RS.

METHODS

Study population

This is a cross-sectional study. From July to October 2010, 251 women seeking gynecologic care at the clinic of the Faculty of Medicine – Federal University of Pelotas (UFPel) were sequentially selected. The study was approved by the Ethics Committee of the Faculty of Medicine – Federal University of Pelotas in June 2009, and informed consent was obtained from all participants. All procedures were carried out in accordance with the guidelines of the Helsinki Declaration.

Statistical analyses

Chi-square (χ^2) test was used to evaluate the HPV presence by PCR and nPCR detection techniques. The analysis was performed using SPSS 16.0 software (SPSS, Chicago, IL). Significance was cosidered if the P value was bellow 0.05.

Sample collection and processing

Cervical samples were collected from each patient with a cytobrush, and placed into 1.5 mL Eppendorf tubes containing 300

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mL of Cell Lysis Solution (Puregene™ DNA Extraction Kit, *Gentra Systems* Minneapolis, MN). The tubes were submitted to digestion using 1.5 µL of Proteinase K (10 mg/mL, New England Biolabs, MA), and incubated overnight at room temperature. The genomic material (DNA) was extracted, according to manufacturer specifications. As a control for extracted DNA quality, the human *TP53* gene PCR was performed, using the primers previously described[14]. PCR were performed in a final reaction volume of 12 µL, and was carried out with one cycle 94ºC for 3 min, followed by 40 cycles at 94ºC for 30 sec, 57ºC for 30, 72ºC for 30 sec and a final extension for 3 min at 72ºC (24).

HPV detection by polymerase chain reaction (PCR) method

Were used the MY09/11 external primers (Table 1), previously described by Manos *et al.*[15]. The MY90/11 PCR reaction was performed in a final volume of 25 µL, and the conditions were as follows: 40 cycles of denaturation (1 min at 95ºC), annealing (1 min at 55ºC), and extension (1 min at 72ºC)[16,17]. Cervix carcinoma cell line HeLa was used as positive control, and amplification mix without DNA as negative control.

HPV detection by the nested polymerase chain reaction (nPCR) method

HPV detection was carried out using nested-PCR (nPCR) technique, which is performed in two rounds: the first using MY09/11 primers, described previously. The second used GP5/6 internal primers (**Table 1**), which amplify the 140 pb fragment[18]. The conditions were 40 cycles of denaturation (30 sec at 94ºC), annealing (30 sec at 45ºC), and extension (30 sec at 72ºC) (7). Both PCR reactions were preheated for 9 min at 95ºC, and a final extension for 5 min at 72ºC. All PCR products were visualized on a 2.0% agarose gel with GelRed™ (Biotium Inc., CA)[19].

RESULTS

All 251 samples were amplified for the *TP53* gene, checking DNA quality for HPV detection by PCR and nPCR methods. The HPV-DNA analysis by PCR MY09/11 was observed in 17 samples, whereas nPCR technique found HPV positivity in more 58 samples (24.8%) shown in **Table 2**, increasing up to 4 times the detection of viral DNA (p < 0.001). The comparative test showed 100% sensitivity in conventional PCR, and a specificity of 75.2% when compared to nPCR (**Table 3**).

Table 1 – General primers sequences for HPV DNA detection

Primer	Sequences* (5'-3')	Size (bp)
MY11	GCMCAGGGWCATAAYAATGG	~ 450[17]
MY09	CGTCCMARRGGAWACTGATC	
GP5	TTTGTTACTGTGGTAGATAC	~ 140[19]
GP6	GAAAAATAAACTGTAAATCA	

* M = A+C; R = A+G; W = A+T; Y = C+T.

Table 2 – Comparison between PCR MY09/11 and nPCR MY09/11 and GP5/6 methods

	nPCR MY09/11 e GP5/6		
PCR MY09/11	Negative (%)	Positive (%)	Total (%)
Negative	176 (75.21)	58 (24.8)	234 (100.00)
Positive	0 (0.00)	17 (100.00)	17 (100.00)
Total	176 (70.12)	75 (29.9)	251 (100.00)

Qui-square $(\chi^2)^{(1)}$ = 42.7916 Pr = < 0.001.

Table 3 – Sensitivity and specificity of MY09/11 PCR compared with nPCR

PCR MY09/11	nPCR Negative	nPCR Positive
Positive	0%	100.0%
Negative	75.2%	24.8%
Total	176	75

Qui-square (χ^2). p < 0.001.

DISCUSSION

There are several comparative studies of different methods for HPV-DNA detection. Husnjak *et al.*[17], also with cervical samples, using conventional PCR with primers MY09/11 compared with nPCR, observed 38.8% of increase in the rate of positivity for HPV DNA by nPCR method, showing that it is more effective in HPV detection. Evander *et al.*[20] reported 5,9% of HPV-DNA detection by the MY09/11 PCR technique. When the nPCR with MY09/11 and GP5/6 was performed, the rate of HPV detection increased to 20,3%, suggesting that GP5/6 primers may result in increased amplification efficiency for not contain any degenerate bases, and for cover a small region compared with MY09/11 primers. However, in a similar study, the authors do not find significant difference: the HPV-DNA detection with MY09/11 primers was 45,2%, and 42,8% for GP5+/6+ primers[12] (Qu et al., 1997).

According to Demathe *et al.*[21], the variations of HPV-DNA detection suggest a potential difference in the ability to amplify fragments of different sizes and specific HPV types, in accordance with the methods of DNA detection used, and also the types of material (smears, frozen material, paraffin or formalin embedded), anatomical location, population issues and the design of primers. The authors evaluated samples from lip squamous cell carcinoma, and observed that the use of nPCR increased by up to 6 times the rate of HPV DNA detection, when compared to MY09/11 PCR.

This study aimed to compare the detection rate of HPV-DNA by PCR method through MY09/MY11 oligonucleotides and nPCR through MY09/MY11 oligonucleotides in the first stage and GP5+/GP6+ in the second stage. This results suggests nPCR technique for HPV-DNA detection is as an alternative to early identification of women at high risk for CC development, showing that the nPCR MY09/11 followed by GP5/6 technique is the most sensitive method to detect HPV DNA from cervical specimens. Moreover, it emphasizes the importance of molecular diagnostic methods as a complementary tool to conventional preventive screenings.

CONCLUSION

The increase in HPV DNA detection from 6.8 to 29.9% suggests that the technique of nPCR MY09/11 followed by GP5/6 is the most sensitive method to detect HPV DNA from cervical specimens.

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Conflict of interest

The authors declared no conflict of interest

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