

# Human papillomavirus detection and typing using a nested-PCR-RFLP assay

## ABSTRACT

**Background:** It is clinically important to detect and type human papillomavirus (HPV) in a sensitive and specific manner. **Objectives:** Development of a nested-polymerase chain reaction-restriction fragment length polymorphism (nested-PCR-RFLP) assay to detect and type HPV based on the analysis of L1 gene. **Methods:** Analysis of published DNA sequence of mucosal HPV types to select sequences of new primers. Design of an original nested-PCR assay using the new primers pair selected and classical MY09/11 primers. HPV detection and typing in cervical samples using the nested-PCR-RFLP assay. **Results:** The nested-PCR-RFLP assay detected and typed HPV in cervical samples. Of the total of 128 clinical samples submitted to simple PCR and nested-PCR for detection of HPV, 37 (28.9%) were positive for the virus by both methods and 25 samples were positive only by nested-PCR (67.5% increase in detection rate compared with single PCR). All HPV positive samples were effectively typed by RFLP assay. **Conclusion:** The method of nested-PCR proved to be an effective diagnostic tool for HPV detection and typing.

**Keywords:** DNA probes, HPV; polymerase chain reaction; polymorphism, restriction; fragment length.

## INTRODUCTION

Cancer of the uterine cervix is one of the most important diseases among women worldwide, with approximately 500,000 new cases and 260,000 deaths per year.<sup>1</sup> In Brazil, the National Cancer Institute (INCA) estimates 18,430 new cases for this current year.<sup>2</sup>

The infection with certain types of human papillomavirus (HPV) is recognized as a causal factor required for the development of cervical cancer.<sup>3</sup> This virus belongs to the Papillomaviridae family and is further classified in 12 *Papillomavirus* genera infecting different animal species. Five of this genera (alfa, beta, gama, mu and nu) can infect humans and are classified according to the oncogenic potential in high (16, 18, 26, 30, 31, 33, 34, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 67, 68, 69, 70, 73, 82) and low-risk types (6, 11, 13, 32, 42, 43, 44, 54, 55, 61, 71, 72, 74, 81, 83, 84, candHPV85).<sup>4</sup>

Molecular methods to detect HPV-DNA (mainly the oncogenic types) have already been developed and are currently used in clinical laboratories. The precise identifica-

tion of viral types is essential for clinical assessment and monitoring of cervical diseases in women.<sup>5</sup> The Hybrid Capture II (Qiagen) is a method widely used in many countries and is able to detect 18 HPV types. This method is based on a non-radioactive signal amplification method that uses two sets of RNA probes, one for low-risk (6, 11, 42, 43 and 44) and another for high-risk types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68). Despite being approved by the Food and Drug Administration (FDA), Hybrid Capture II has some important limitations, as it does not detect some HPV types, does not identify the specific type of HPV, is less sensitive than PCR and presents cross-reactivity of the RNA probes (for low and high risk), reducing the clinical relevance of a positive result.<sup>6-10</sup>

Alternatively, there are several PCR methods to detect HPV-DNA. The use of primers targeted to highly conserved consensus sequences of the virus (mainly in the L1 region) allows simultaneous amplification of a large number of mucosal HPV types. The majority of the PCR methods use single PCR

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amplification with two pairs of consensus primers: MY09/11 or GP5+/6+.<sup>11,12</sup> Primers pair MY09/11 has been largely used in clinical and epidemiological studies.<sup>13-16</sup> One of the main advantages of this primers combination is the possibility of determining the HPV type using different procedures: restriction fragment length polymorphism (RFLP) assay, sequencing, and micro-array technology.<sup>10,17-20</sup>

Nested-PCR techniques have proven to be more sensitive for the detection of HPV than PCR methods based on only one amplification reaction.<sup>21,22</sup> A nested-PCR method using MY09/11 and GP5+/6+ primers has been used in large epidemiological studies for the sensitive detection of HPV in cervical samples.<sup>16</sup> However, the nested-PCR techniques previously described require additional sequencing or hybridization procedures to accurately identify the HPV type.<sup>21,23</sup> This study aimed to develop and validate a highly sensitive nested-PCR-RFLP methodology for HPV detection and typing easily applied in limited-resource laboratories.

## MATERIALS AND METHODS

The project "Evaluation of the use of HPV-DNA detection in screening for precancerous lesions and cervical neoplasia in an urban center in Southern Brazil" was approved by the Ethics Committee of Universidade de Cruz Alta under the protocol number 078.0.417-09.

### Clinical samples

The first group of samples included a panel of 44 clinical samples, positive and negative for HPV, previously analyzed in a molecular diagnostic laboratory. These samples were previously detected by the PCR-RFLP method and presented the following HPV types: 6, 11, 16, 33, 53, 54, 56, 58, 61, 62, 66, 70 (CP 141), 83 (MM7) and 84 (MM8). The second group included 84 samples obtained prospectively from women seeking routine care in a screening program for cervical cancer in a public health service in southern Brazil.

All the samples were collected by scraping the ecto and endocervix with individual brushes. Each brush was packed in a modified buffer solution (EDTA pH 8.0 0.01 M, SDS 0.03 M) and conserved at -20°C until analysis.

### HPV DNA sequences analysis

Published DNA sequences of the main mucosal HPV types (6, 11, 13, 16, 18, 26, 30, 31, 32, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 61, 66, 67, 68, 69, 70, 71, 72, 73, 74, 81, 82, 83, 84, 90, cand85, cand86, cand87, cand 89, cand91 - all of them classified in the Alpha-papillomavirus genus) were obtained from the GenBank database (<http://www.ncbi.nlm.nih.gov/Genbank/>). These sequences were edited and aligned using EditSeq and MegAlign softwares from the DNASTAR package (Lasergene Inc., USA), respectively. DNA segments of the L1 gene were selected and

compiled in a document file for primer design. The PCR primers pair was selected directly in the aligned sequences and the hybridization temperature was estimated using the PrimerSelect software also from the DNASTAR package (Lasergene Inc., USA).

### DNA extraction

Total DNA of the clinical samples was purified by a standard silica/GuSCN-based procedure.<sup>24</sup> Briefly, 100 µL of the liquid sample was added to 900 µL of lysis buffer (GuSCN 5 M, Tris-HCl 0.1 M, EDTA 0.5 M and Triton X-100) and then incubated at room temperature for 10 min. After, 20 µL of silica suspension was added to each sample, mixed and centrifuged at 10,000 rpm for 30 sec. The pellet was washed twice with 1.0 mL washing buffer (GuSCN 5 M and Tris-HCl 0.1 M), twice with 75% ethanol and once with acetone. The silica suspension was dried at 56-60°C for 10 min. DNA was eluted with 50 µL of TE buffer and the solution was separated from the silica particles centrifuging at 10,000 rpm for 3 min to use as template in PCR reaction.

### HPV detection by PCR and nested-PCR assays

HPV-DNA was analyzed by PCR (with one amplification reaction) and nested-PCR assays. PCR was carried out in a total volume of 30 µL using 2 µL of template DNA, 1X PCR buffer (10 mM Tris-HCl pH 8.5, 50 mM KCl), 1.5 mM MgCl<sub>2</sub>, 0.0625 mM of each deoxynucleotide triphosphate (dATP, dTTP, dCTP, dGTP), 0.5 µM of each consensus primers MY09/MY11 and 1.6 U of Taq DNA polymerase (Cenbiot Enzimas, RS, Brazil). Thermal cycling was performed in a PE Applied Biosystems 9700 Sequence Detector System with the following program: 4 min at 95°C, 35 cycles of 30 sec at 94°C, 30 sec at 56°C and 30 sec at 72°C, with final extension step at 72°C for 8 min.

Nested-PCR assay was performed in two consecutive amplification reactions. The first reaction was carried out in a total volume of 30 µL using 2 µL of template DNA, 1X PCR buffer (10 mM Tris-HCl pH 8.5, 50 mM KCl), 1.5 mM MgCl<sub>2</sub>, 0.0625 mM of each deoxynucleotide triphosphate (dATP, dTTP, dCTP, dGTP), 0.5 µM of each primer, 1 U of Taq DNA polymerase (Cenbiot Enzimas, RS, Brazil). Amplification was performed in a PE Applied Biosystems 9.700 with this program: 1 cycle at 95°C for 3 min and 20 cycles at 95°C for 20 sec, 40°C for 40 sec and 72°C for 1 min, followed by a final extension cycle at 72°C for 5 min. The second reaction was also carried out in a volume of 30 µL using 2 µL of amplified DNA, 0.25 µM of the primers (MY09/11) and the same reagents and concentrations described above. Thermal cycling was performed in the same equipment with the following steps: 1 cycle at 95°C for 3 min, 35 cycles at 95°C for 20 sec, 55°C for 40 sec and 72°C for 1 min, and a final extension cycle at 72°C for 5 min.

Positive and negative samples were added as controls in all experiments for quality control. The amplified DNA products and molecular weight markers were then submitted to electrophoresis in 10% polyacrylamide gels at 65 V for 1 hour. Each gel was stained with silver nitrate and positive/negative results were evaluated based on the presence of fragments of the expected size (~450 bp).

### HPV typing by RFLP assay

All HPV positive samples were typed by RFLP analysis. Each restriction reaction was performed separately in a final volume of 10  $\mu$ L, using 2  $\mu$ L of MY09/11 PCR product, 1  $\mu$ L of 10X recommended restriction buffer and 10 units of the following restriction endonucleases: *Pst*I (New England Biolabs, USA), *Hae*III (New England Biolabs, USA), *Dde*I (New England Biolabs, USA), *Rsa*I (Promega, USA) and *Hinf*I (New England Biolabs, USA), according to the manufacturer's instructions. Reactions were carried out at 37°C for 1 hour. Digested products were separated on 10% polyacrylamide gels by electrophoresis together with a 50 bp DNA molecular weight marker (Promega, USA), and after stained with silver nitrate.

### Confirmation of RFLP analysis by sequencing

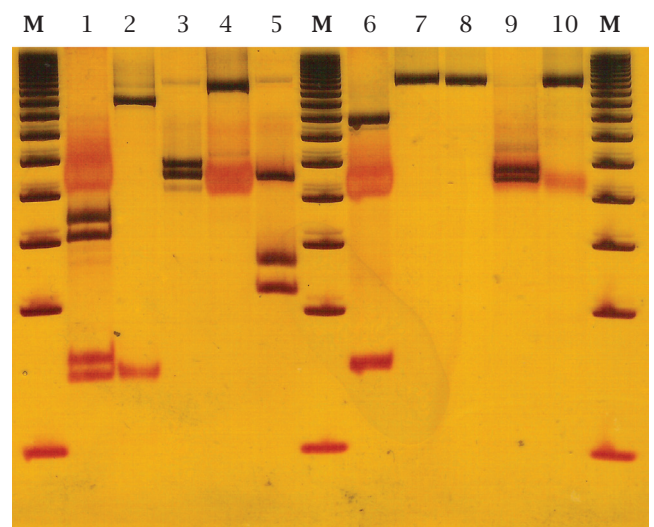
PCR products were sequenced to confirm the RFLP results. Forward and reverse sequencing reactions were carried out using 15 to 30 ng of template DNA from PCR amplimers, 3.2 pmol of the MY09 and MY11 primers (respectively) and 4  $\mu$ L of reagent BigDye Terminator v3.1 Cycle Sequencing (Applied Biosystems Inc., Norwalk, CT, USA) in a final volume of 20  $\mu$ L. The sequencing rounds were performed in the thermocycler Veriti 96 (Applied Biosystems Inc., Norwalk, CT, USA) with the initial step of 95°C for 3 min followed by 40 cycles of 95°C for 10 sec and 60°C for 240 sec. The samples were purified by ethanol/EDTA/sodium acetate protocol and the precipitated DNA products were diluted in 10  $\mu$ L of formamide Hi-Di, denatured (95°C for 2 min) and injected in the automated DNA sequencing ABI 3130 XL Genetic Analyzer (Applied Biosystems Inc., Norwalk, CT, USA). The sequence data were collected using the Data Collection program v1.0.1 with the parameters Dye Set "Z". Quality analysis was performed using the Sequencing Analysis v.5.3.1 software by evaluating the main technical parameters as raw data, electropherogram and quality value of sequenced bases (Applied Biosystems Inc., Norwalk, CT, USA). The nucleotide sequences of the same amplicon (primers sense and antisense) were edited and assembled using SeqMan software (DNASTar, Madison, Wisconsin, USA). The nucleotide sequences of the samples were aligned using the MegAlign program (DNASTar, Madison, Wisconsin, USA) and types were deduced from the phylogenies generated by the program itself. Further, data obtained were compared with sequences available in GenBank database ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) using BLAST tool.

## RESULTS

### Primers and nested-PCR-RFLP design

After alignment of the L1 HPV sequences obtained in GenBank, conserved sequence regions (upstream to MY09 primer and downstream to MY11 primer) were selected and analyzed in PrimerSelect software (Lasergene Inc., USA). Considering a fitness combination for a primers pair and detection of all HPV types, the following sequences were selected: 5'-CAA WTR TTY AAT AAR CCW TAT TGG-3' (primer SB01) and 5'-AAA AAY TTY CGW CCM ARR GG-3' (primer SB02). PCR product is approximately 495 bp for the majority of HPV types. Using this primers pair, a novel detection and typing procedure based on nested-PCR-RFLP assay was designed. The whole procedure consisted in a first amplification by PCR with low stringent condition (annealing temperature of 40°C), a second amplification by PCR using MY09/11 primers pairs (nested-PCR) and typing by restriction fragment length polymorphism (RFLP) assay.

This procedure was used to detect and type HPV in DNA samples extracted from two previously analyzed clinical samples with positive results for types 6 and 16. In both samples, the expected ~450 bp fragment of L1 gene was successfully amplified by nested-PCR assay. Further, RFLP assay demonstrated the same pattern of the two types previously found (Figure 1). To confirm the specificity of the assay, the nested-PCR assay was also performed with DNA extracted from negative HPV cervical samples. HPV specific fragments were not detected.



**Figure 1:** Restriction enzyme patterns of MY09/11 amplicon from HPV types 6 and 16. Lanes M, 50 bp ladder size marker; lanes 1 to 5, HPV 6 digested with *Rsa*I, *Dde*I, *Hinf*I, *Pst*I and *Hae*III; lanes 6 to 10, HPV 6 digested with *Rsa*I, *Dde*I, *Hinf*I, *Pst*I and *Hae*III.

### Evaluation of the nested-PCR-RFLP assay with clinical samples

The nested-PCR-RFLP assay was compared to MY09/11 PCR assay using two groups of cervical samples. The first group had 44 previously analyzed cervical samples of a service laboratory, 24 HPV-DNA positive and 20 negative by MY09/11 PCR. A total of 36 samples were positive and 8 negative using the nested-PCR assay. All the 24 positive samples by MY09/11 PCR were also positive by nested-PCR. In addition, 12 samples were exclusively positive for HPV by the nested-PCR assay (Table 1). Afterwards, 84 consecutively collected cervical samples (second group), obtained from women who underwent routine examination for cervical cancer screening in a public health service in a city (Cruz Alta) of Rio Grande do Sul State (Southern Brazil), were analyzed by both procedures (MY09/11 PCR and nested-PCR assays) in a blind fashion experiment. Again, all the positive samples by PCR (13) were also positive in the nested-PCR and other 13 samples were exclusively HPV positive using the nested-PCR assay (Table 1).

**Table 1. Results of HPV-DNA detection using MY09/11 PCR in comparison to nested-PCR assays in two groups of cervical samples (pre-analyzed and prospectively collected)**

Nested-PCR	MY09/11 PCR		
	Positive n = 37	Negative n = 91	Total n = 128
First group			
Positive	24	12	36
Negative	0	8	8
Total	24	20	44
Second group			
Positive	13	13	26
Negative	0	58	58
Total	13	71	84

In the analysis by RFLP assay, 23 different HPV types were identified (12 high-risk and 11 low-risk). Importantly, two high-risk types (HPV 45 and HPV 73) and three low-risk types (HPV 44, HPV 71 and HPV 81) were detected only by nested-PCR-RFLP assay (Table 2). Mixed infections were identified in 14 samples and the HPV types (two in all samples) of each case were efficiently discriminated by the RFLP assay. DNA sequencing was consistent with the pattern obtained by RFLP assay in all cases with only one HPV type in the sample. In the 14 mixed infection cases, DNA sequencing identified one of the types detected by RFLP assay.

### DISCUSSION

The wide variation of HPV types and the occurrence of multiple infections by this virus require reliable methods for HPV detection and typing. These methodologies are essential for patient management by the clinician and even epidemiological studies. In the near future, HPV detection and typing will also be important in the screening and monitoring of women that use specific vaccines or therapies.<sup>25</sup>

HPV detection is based on the analysis of the viral DNA by different methods (hybrid-capture, PCR, hybridization). In the PCR assays currently used, consensus primers (MY09/11, GP5+/6+, PGMV, SPF10) are targeted to the highly conserved L1 region and detect different HPV types.<sup>12,26-28</sup> Previous studies have already demonstrated that the use of primers pair MY09/11 and GP5/GP6 in a nested-PCR assay increases the sensitivity of HPV detection compared with PCR assay.<sup>18,21,25,29,30</sup> However, all of these studies used an additional sequencing and/or hybridization step of the amplification product for HPV typing.<sup>23</sup>

In this study, the development of a methodology based on nested-PCR technique, using an external primers pair in a first amplification and the "classical" primers pair MY09/11 in a second reaction, enabled the sensitive HPV detection in cervical samples. This was possible with the design of a new degenerated primers pair external to the region of the MY09/11 amplified product and the use of low stringent condition (annealing temperature of 40°C),

**Table 2. HPV types identified by RFLP in the positive samples after amplification with MY09/11 PCR and nested-PCR assays**

	High-risk types (n)	Low-risk types (n)
MY09/11 PCR and nested-PCR (n = 37)	16 (6) <sup>b</sup> , 18 (1) <sup>b</sup> , 33 (6) <sup>b</sup> , 35 (1), 52 (2) <sup>b</sup> , 53 (3) <sup>b</sup> , 56 (2) <sup>b</sup> , 58 (2) <sup>b</sup> , 66 (4) <sup>b</sup> , 70 (1)	6 (2) <sup>b</sup> , 11 (4) <sup>b</sup> , 42 (2), 54 (5) <sup>b</sup> , 61 (2) <sup>b</sup> , 62 (1), 83 (1) <sup>b</sup> , 84 (4) <sup>b</sup>
Only by nested-PCR (n = 25)	16 (2) <sup>b</sup> , 35 (1), 45 <sup>a</sup> (4), 52 (1), 53 (1) <sup>b</sup> , 58 (2), 66 (1), 70 (3), 73 <sup>a</sup> (2) <sup>b</sup>	6 (1) <sup>b</sup> , 11 (1), 42 (1), 44 <sup>a</sup> (1), 54 (2), 62 (1), 71 <sup>a</sup> (1) <sup>b</sup> , 81 <sup>a</sup> (1) <sup>b</sup> , 84 (1)

<sup>a</sup> HPV types identified only by nested-PCR; <sup>b</sup> HPV types found in the 14 samples with mixed infection.

aiming the amplification of all cervical HPV types. In the analysis of pre-selected clinical samples (mostly positive) and cervical samples of a women group (mostly negative), an estimated 67.5% of increase in the rate of HPV detection was obtained as compared to MY09/11 PCR. Husnjak et al.,<sup>31</sup> comparing different techniques of PCR to detect HPV DNA in cervical samples, has reported an increase of 38.8% detection rate using the method of nested-PCR (GP5/GP6) compared to single PCR amplification. Furthermore, in our study, all the 37 positive samples by PCR with MY09/11 primers were also detected by the nested-PCR assay (there were no cases of HPV detected by MY09/11 PCR and not detected by nested-PCR).

Considering PCR with MY09/11 primers as the “gold standard”, the clinical sensitivity and specificity were 100% and 72%, respectively. But it is important to consider that PCR with MY09/11 primers has clearly a lower analytical sensitivity than the nested-PCR assay developed in our study and this is the probable reason for the low clinical specificity of the assay. Previous works have already demonstrated a higher analytical sensitivity for other nested-PCR assays.<sup>25,30</sup> We further classified the samples into two groups (positive by both procedures versus positive only by nested-PCR) and observed different patterns of HPV types and frequency by RFLP and sequencing. Some types were detected only by nested-PCR, as for example the high-risk types 45 and 73 and the low-risk types 44, 71 and 81 (Table 2). This reinforces that the samples positive only in the nested-PCR assay are probably not false-positive results, but due to the higher analytical sensitivity of this assay. Other studies have also used nested-PCR assays to maximize the detection of HPV in different populations.<sup>16,21,23,32</sup>

Besides the increase in the analytical sensitivity, another advantage of this new methodology is the possibility to perform HPV typing by RFLP assay, an easier and cheaper methodology than sequencing. In our study, the nested-PCR-RFLP assay detected precisely low (11, 42, 44, 54, 62, 70, 81, 84) and high-risk types (16, 18, 33, 35, 45, 52, 53, 58, 73). All these results were confirmed by DNA sequencing. These findings are similar to those reported by Aedo et al.,<sup>18</sup> which identified 13 different types in samples tested with nested-PCR-RFLP in Chile. Similarly, Camara et al.<sup>33</sup> using the methodology of PCR-RFLP identified 13 viral types in women of the Distrito Federal, Brazil.

The methodology presented in this paper combines an easy and fast DNA extraction procedure, a sensitive nested-PCR amplification technique and a RFLP procedure that identify the main cervical HPV types. The procedure is suitable for the detection and characterization of HPV-DNA in public health programs to screen this virus in the population and would be also a useful tool for HPV epidemiological control, determining the circulating types in different geographical regions. Furthermore, it could be easily per-

formed in limited-resource laboratories, not requiring additional sequencing or hybridization procedures (and all the equipment necessary) to accurately identify the HPV type.

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