

# Identification of Human Papilloma Virus 16 in Uterine Cervix Smears

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**ABSTRACT.** Epidemiological studies have identified human papilloma virus (HPV) infection as the main risk factor associated with development of cervical cancer. It has been found that 80 to 100% of advanced neoplasms and cervical invasive carcinomas contain only a few HPV types. Type 16 is the most prevalent of the invasive carcinomas (30-60%). It has therefore been suggested that follow-up and treatment of infected patients must be directed against the specific infective virus. Our objective was to develop a rapid method of identifying HPV 16 in cervical smears. To do this, a type-specific PCR amplification stage has been designed for the region corresponding to the HPV 16 E6 oncogene, followed by product confirmation by restriction enzyme digestion analysis. This method has been applied to fifteen fresh cytological cervical smear samples, obtained from patients infected with HPV 16, which were previously diagnosed by HSIL. The CaSki cell line genome carrying HPV 16 was used as the positive control and the HeLa cell line genome, normal human lymphocyte genome and two cytological samples infected with HPV 18 and 6, respectively were used as negative controls. A specific 140 bp amplification product was obtained from the HPV 16 E6 oncogene in all samples analyzed and in the positive control. Further enzyme restriction with *Rsa* I produced the expected cutting pattern of two fragments consisting of 90 and 50 bp. Negative controls and the samples infected with HPV 18 and 6 did not produce a specific amplification product. This method could be used as a rapid method of identifying HPV 16 in fresh cervical samples.

**Key words:** HPV 16, genotyping, cervical cancer.

**RESUMEN.** Epidemiológicamente se ha identificado a las infecciones por Virus Papiloma Humano (HPV) como el mayor factor de riesgo asociado al desarrollo de cáncer de cérvix. Entre el 80 y 100% de las lesiones neoplásicas avanzadas y los carcinomas invasores de cérvix contienen sólo unos pocos tipos de HPV. El tipo 16 el más prevalente en carcinomas invasores (30-60%). El seguimiento de pacientes infectadas y la conducta terapéutica se sugiere debe orientarse según el tipo viral infectante. Nuestro objetivo fue desarrollar una metodología rápida para la tipificación de HPV 16 en hisopados de lesiones de cérvix. Para ello se diseñó una PCR tipo-específica a partir de una región del oncogén E6 del HPV 16 seguida de la confirmación del producto obtenido mediante análisis de la restricción enzimática. Con este método, se analizaron quince muestras citológicas frescas obtenidas de hisopados de cérvix de pacientes infectadas por HPV 16 y con diagnóstico HSIL. Se emplearon como control positivo genoma de la línea celular CaSki que porta HPV 16 integrado y como controles negativos genomas de la línea celular HeLa, de linfocitos humanos normales y de dos muestras citológicas infectadas con HPV 18 y 6. Se obtuvo el producto específico de amplificación (140 pb) del oncogén E6 del HPV 16 en todas las muestras bajo análisis y en el control positivo. La posterior restricción enzimática con *Rsa* I produjo el patrón de corte esperado, dos fragmentos de 90 y 50 pb. Los controles negativos y las muestras infectadas con HPV 18 y 6 no mostraron producto de amplificación específico. Este método puede ser de utilidad para la tipificación de infecciones por HPV 16 en muestras frescas de lesiones de cérvix.

**Palabras Clave:** HPV 16, genotipificación, lesiones de cérvix.

## INTRODUCTION

Various epidemiological and clinical studies have established a strong correlation between genital infection with the human papilloma virus (HPV) and development of uterine cervix cancer.<sup>1,9,15,24</sup> This type of neoplasm represents approximately 6% of all human cancers and is

the second most common form found in women.<sup>6,10,18,21,23</sup>

The application in recent years of molecular biological techniques has resulted in the exponential growth of knowledge related to the biology and pathogeny of HPV. To date, 78 different viral types causing human infection in the skin and mucosae are known.<sup>5,8</sup>



It has been found that 80 to 100% of advanced neoplasms and invasive cervical carcinomas are caused by only a few types of mucosotropic HPV. From the clinical point of view these have been classified as "high" [types 16 and 18], "intermediate" [types 31, 33 and 35] or "low" [types 6 and 11] risk, and it is type 16 that is the most prevalent in invasive carcinomas (30-60%). It has therefore been suggested that patient follow-up and therapy should be directed against the specific type of viral infection.<sup>18</sup>

Viral identification techniques are available commercially, such as *in situ* hybridization, Southern blot, PCR and the hybrid capture system. However, all these methods have different experimental reliabilities, based on their ability to generate consistent and comparable results when applied to a large number of clinical samples analyzed over a prolonged period of time.<sup>6,11,12</sup> The most sensitive technique available is the polymerase chain reaction (PCR). This technique uses generic primers recognizing consecutive sequences present in the genomes of the majority of infective mucosotropic viruses, followed by genotyping of the amplification product by membrane hybridization using type-specific probes.<sup>3,14</sup> This experimental approach has produced important epidemiological findings worldwide and has provided reliable and comparable information.

Studies carried out in different regions of Argentina have shown that local epidemiological patterns do not substantially differ from those found in other countries. That is, in Argentina HPV type 16 is most commonly found in cervical cancers and also in pre-cancerous states.<sup>16,22</sup>

Molecular analysis of the genetic expression of this viral type has shown that the proteins originating from the initial regions of the genome (genes E6 and E7) immortalize human keratinocytes *in vitro*, and that these regions are probably the main cause of malignant transformation of the cervical epithelium by direct interaction with cell cycle regulatory proteins, in particular p53 and pRB. Furthermore, continuous expression of E6 and E7 oncoproteins is required to maintain the transformed phenotypes.<sup>4,19,20</sup>

The presence of persistent HPV type 16 infection in association with additional risk factors such as multiple sexual partners, sexual promiscuity, hormonal factors and genetic predisposition, are determinant elements in the evaluation of pre-cancerous progression. Hence, identification of HPV 16 in cervical cancer is of particular interest from the health care point of view.<sup>2</sup>

Taking the above factors into consideration, our objective was to develop an alternative quick and specific method to identify HPV 16 in cervical cancer. We have therefore designed a mixed technique that includes a type-specific PCR amplification stage of the HPV 16 E6 oncogene region, followed by confirmation of the product obtained by enzyme restriction and analysis of the running profiles of cut fragments in polyacrylamide gels.

## MATERIALS AND METHODS.

**Samples and controls.** Total DNA, isolated from fifteen fresh cervical smear samples, was analyzed. Samples were obtained from patients classified by high scaly intra-epithelial lesion (HSIL),<sup>13</sup> who were attending the Cervical Pathology Unit in the Central Provincial Hospital Dr. Madariaga, Posadas City, Misiones Province, Argentina, following informed patient consent. All samples had previously been identified as single HPV 16 infections using the PCR amplification technique with GP5+ and GP6+ primers and dot hybridization with type-specific probes.<sup>3</sup>

The positive control was the CaSki cell line genome carrying HPV 16 and the negative controls were the HeLa cell line genome with integrated HPV 18 and the normal human lymphocyte genome (male cells).

**Total DNA isolation and purification.** The cell pellet obtained from cervical brush smears was re-suspended and washed in a solution of 5 mM EDTA, 0.5 % SDS and 10mM Tris (pH 8.0). This was followed by proteolytic digestion in 200 µl/ml of proteinase K for 2 h at 56 °C ± 1 °C. Total DNA was purified by extraction with isoamyl alcohol-phenol-chloroform-alcohol and precipitated with absolute ethanol at -20 °C. The final re-suspension was made in sterile distilled water.

**Type-specific PCR amplification.** The 140 bp fragment of the HPV 16 E6 oncogene genome (bases 109 to 248), was amplified by PCR using the following primers (Forslund, Lindqvist *et al.* 1997):

**E6+** TCA GGA CCC ACA GGA GCG ACC [Genomic site 109-129 bp]

**E6-** CCC GAA AAG CAA AGT CAT ATA C [Genomic site 248-227 bp].

The reaction mixture was made up to a total volume of 50 µl, containing: Total DNA from samples or controls, 1X buffer (10 mM Tris ClH pH 8.8; 50 mM ClK; 2 mM MgCl<sub>2</sub>, 1ml/l Triton X-100), 0.2 mM of dNTPs (dATP, dCTP, dGTP and dTTP), 0.2 µM of each primer (E6+ and E6-) and 2.5 U of Taq DNA polymerase (Promega).

The PCR reaction was started using the "Hot start" technique and was continued using the following thermic cycle program, (Techne PHC3): Denaturing for 40 cycles at 94 °C for 45 s; hybridization at 57 °C for 30 s and extension at 72 °C for 40 s.

**Enzyme restriction.** 20 µl of the type-specific amplification reaction mixture were digested with 10 IU of the *Rsa* I restriction enzyme for 2 h, according to the manufacturer's recommendations (Promega). The digestion products were then analyzed following polyacrylamide gel electrophoresis.

**Electrophoresis.** 10 µl of the restriction enzyme reaction mixture were separated by electrophoresis in a 12 % polyacrylamide gel (Biorad) with TBE buffer. The separated products were stained with ethidium bromide and visualized by UV transillumination and photographed with Gelcam Polaroid equipment.

## RESULTS

Type-specific amplification of a region of the HPV type 16 E6 gene using primers described in this work generated the following sequence of 140 base pairs (Fig 1.):

5' **T C A G G A C C C A C A G G A G C G A C C C A** -  
GAAAGTTACCACAGTTATGAGCTGCAAACA  
TACATGATATAATATTAGAATGTGTGTACGCAAC  
AGTTACTGCGACGTGAGGTA **TATGACTTTGCTTT**  
**TCGGG** 3'.

Under the conditions described, this pair of primers did not hybridize to the other common types of mucosotropic HPV (types 18, 31, 33, 35, 6 or 11), and hence an amplification product was not sequenced.

Enzyme restriction of specific amplification products with the *Rsa* I enzyme, produced two fragments consisting of 90 and 50 base pairs, respectively (Fig. 2).

Based on sequence analysis this result was expected. The sequence carries a single recognition site for this enzyme (5' **GTAC** 3'):

5' **T C A G G A C C C A C A G G A G C G A C C C A** -  
GAAAGTTAACACAGTTATGCACAGAGCTGCAA  
CAACCTATACATGATATAATATTAGAATGTGT**GT**  
**ACTGCAAGCAACAGTTACTGGACGTGAGGTATAT**-  
**GACTTTGCTTTTCGG** 3'

## DISCUSSION

A 140 bp fragment of the HPV 16 E6 oncogene was amplified by PCR (bases 109 to 248). The specific product was obtained from the positive control and from each of the fifteen clinical samples previously identified by conventional methods as infected with HPV type 16. The controls using other viral types did not generate the amplification product.

Later enzyme restriction of the sequence with *Rsa* I, produced the expected pattern. Fragments of 90 bp and 50 bp were generated, which were detected by polyacrylamide gel electrophoresis. The completion of the method for identifying HPV 16, starting from total DNA derived from a uterine cervix smear, takes about 8 h. This is in contrast with conventional amplification techniques followed by type-specific hybridization, which require more time

From these preliminary results we conclude that the method described may be useful as a rapid method of identifying HPV 16 infections in fresh uterine cervix samples.

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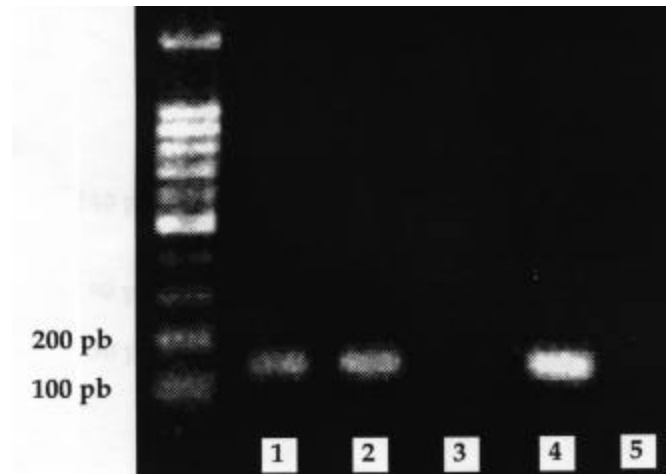


Fig. 1. Electrophoresis in 1 % agarose gel in TAE buffer of the PCR amplification products, using the E6+ and E6-primers. A single product of approximately 140 bp is shown in the clinical and positive control samples. 1, positive control, CaSki genome partially digested with *Rsa* I; 2, positive control, CaSki total digest; 3 and 4, clinical samples infected with HPV type 16; 5, negative control.

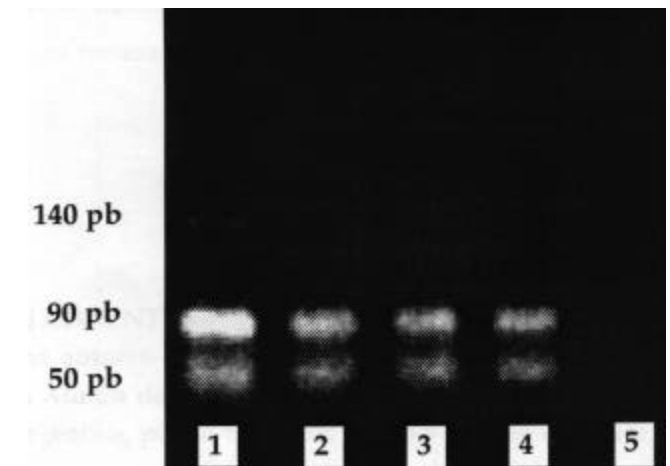


Fig. 2. Electrophoresis of PCR products digested with *Rsa* I enzyme, in 12 % polyacrylamide gel with TBE buffer. In lane 1 the remains of the original 140 bp type-specific amplification sequence are seen and the two restriction products of 90 and 50 bp, respectively. All clinical samples analyzed showed a similar pattern. 1, positive control, CaSki genome partially digested with *Rsa* I; 2, positive control, CaSki total digest; 3 and 4, clinical samples infected with HPV type 16; 5, negative control.

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