A system for the detection and typing of Human Papillomavirus of the lower genital tract: *In situ* hybridization screening and polymerase chain reaction confirmation.

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**Key words:** Diagnostic, typing, HPV, *in situ* hybridization, polymerase chain reaction, lower genital tract.

**Abstract.** We developed a simplified and non-isotopic *in situ* hybridization procedure for the detection of Human Papillomavirus (HPV) in routine Papanicolaou cervical smears. The assay involves one oligonucleotide (malignant probe) which recognizes high risk HPV 16 and 18, and another which detects HPV 6 and 11 (benign probe). We adapted the system to fulfill the requirements of gynecologists and cytologists, assimilating their protocols and simplifying the *in situ* hybridization assay. When we compared the detection levels reached by the *in situ* hybridization versus a ladder PCR assay in 156 clinical samples, original designed for this work, the kappa coefficient between both methods is 0.945, indicating a strong agreement between the ISH and the PCR assays.
Sistema de detección y tipificación de Papilomavirus del tracto genital inferior: Hibridización in situ y confirmación con reacción en cadena de la polimerasa. 

**Palabras clave:** Diagnóstico, tipificación, VPH, hibridización in situ, reacción en cadena de la polimerasa, tracto genital inferior.

**Resumen.** Se desarrolló un procedimiento de Hibridación in situ no-isotópico y simplificado para la detección de papilomavirus humano (HPV) en citologías de rutina de Papanicolaou. El ensayo involucra un oligonucleotido (sonda maligna) el cual reconoce a los VPH de alto riesgo: 16 y 18, y otra que detecta VPH 6 y 11 (sonda benigna). Se adaptó completamente el sistema a los requerimientos de ginecólogos y citotécnicos, asimilando sus protocolos y simplificando el ensayo de hibridación in situ. Se compararon 156 muestras clínicas, los niveles de detección tanto en hibridación in situ como en el ensayo de PCR (diseño original para este trabajo), se obtuvieron valores de co-positividad y co-negatividad cercanos a la unidad (> 0,98).


**INTRODUCTION**

The human papillomavirus (HPV) has been associated with the genesis of human cancer (1-3). Out of the 100 identified viral types (4-6), a fourth them preferentially infects the mucosal epithelium lining of the anogenital tract, and types 16, 18, 31, 33, 35 and 45 are considered as high risk viruses by their close association with all grades of squamous intraepithelial lesions (SIL) and invasive cancers (7-9). Types 6 and 11, coexist with benign Condylomata acuminata (venereal warts) and low-grade of SIL (10), and are generally treated as low risk viruses.

The common test for the analysis of HPV-lesions is the Papanicolaou stain, commonly known as the Pap Smear (PS), this method combines simplicity and accuracy, but owing to its low sensitivity it is only reliable on acute viral infections (11). Another handicap of this technique is the lack of reproducibility, the results fluctuate from one laboratory to another, and although efforts are made to refine the staining procedure (12, 13) a standardized protocol is not widely applied. Finally, the PS is not sufficiently predictive on HPV-induced neoplasias. In fact, Lörincz y col. (14) showed that 25% of patients with advanced in situ carcinoma, presented normal PS a few years before diagnosis. Therefore, new diagnostic tools for the early detection of high-risk HPV are needed (15). Such assays will permit the physician to reveal the presence of high-risk HPV when no damage to the host cell is yet apparent, and establish a close surveillance in those patients. The methods develop to detect HPV by immunocytochemistry have single type poor sensitivity and do not discriminate between viral types. Gupta y col. (16) showed that only 57% of the biopsies with clear morphological alterations were positive for this type of test. In addition, a cell culture system to produce large quantities of HPV antigens is not yet available.
The in situ hybridization techniques (ISH) although somewhat laborious, fulfill these needs. Using whole DNA probes and non-radioactive labeling, HPV was detected in infected cells in culture (17-19) and in preserved tissue specimens (20-22).

Recently, with the availability of the DNA sequences of most HPV types, it is possible to choose specific oligonucleotides to perform ISH in PS. The use of oligonucleotides in ISH has several advantages: a) the specificity of the assay can be increased by focusing on specific targets and removing sequences in common with other viral types. b) Smaller DNA probes have a better penetration in cell tissues, and hence, easier protocols can be designed. c) they can be labeled to a very high specific activity, thus improving the assay's sensitivity.

Although the use of polymerase chain reaction (PCR) in the diagnosis of HPV permits the detection of a few viral copies in complex samples (13, 23-25), it does not show the virus location, and demands special skills.

In this work we used a new ISH on PS to detect the most common HPVs infecting the anogenital tract. The assay combines the use of short oligonucleotides labeled to a high specific activity, with a simplified handling of the samples. We also designed a ladder multiplex-PCR protocol to independently assess the presence of HPV in the PS samples examined.

MATERIALS AND METHODS

HPV clones and clinical specimens

Cloned genomes of HPV types 6b, 11, 16, and 18 were a generous gift from Drs. H. zur Hausen and E. de Villiers (Heidelberg, Germany). The present series consisted of 156 clinical samples collected from patients attending the Gynecology Service of a large, urban, municipal oncological reference center, Hospital Oncológico Padre Machado, and from a private clinic at Caracas (Urológico San Román). Cervical scrapes from endo-and exo-cervix were taken with a sterile round-cotton tip, and the adhered cells were circularly spread on a pre-cleaned slide (micro slides, Superfrost, VWR Scientific), covering an area of about 1.5 cm diameter. The cells were fixed and the cotton swab was immersed in an Eppendorf tube containing IX phosphate buffered saline (PBS) and 0.02% sodium azide. The samples were kept at -20°C until use.

Sequence analysis and oligonucleotide designing and selection

We used the software DNAsis v7 (Hitachi) to analyze the HPV sequences retrieved from the GenBank database, and to select the specific oligonucleotides used in the ISH and PCR assays.

From the DNA sequence analysis, we selected oligonucleotide 1 (benign probe): 5´ GAACTTATTACCAGTGTTATACAG G 3´, for the detection of HPV 6 and 11, and oligonucleotide 2 (malignant probe): 5´ ATATCAGATGACGAGRACGAAAAT G 3´ (R being Adenine or Guanine), for HPV 16 and 18. The sequence of oligonucleotide 1 is located within the L1 open reading frame (ORF) of 6b and 11, beginning at positions 6326, respectively. Oligonucleotide 2 is contained within ORF E1 of HPV 16 and 18, beginning at positions 961 and 1007, respectively. To test the specificity of these oligonucleotides, we hybridized them against whole genomic DNAs from HPV 6, 11, 16 and 18, blotted onto nylon filters.

Labeling of oligonucleotides and whole HPV genomes for ISH

We labeled the oligonucleotides probes as follows: to 200 ng of each oligonucleotide, we added a reaction mixture containing 140 mM potassium...
cacodylate, 30 mM tris base pH 7.2, 1 mM CoCl₂, 0.1 mM dithiotreitol (DTT), 0.1 mM digoxigenin-11-dUTP, 25 units of 3’-exo nucleotidyl transferase (Boehringer Mannheim), and adjusted the final volume to 25 µL. The reaction mixture was incubated for 1.5 h at 37°C. When whole viral genomic probes were used, we separated the viral genome-containing inserts from the cloning vectors by restriction enzyme digestion and by agarose gel electrophoresis. The purified inserts were labeled by random priming using digoxigenin-11-dUTP Genius labeling and detection kit (Boehringer Mannheim) according to the manufacturer’s instructions.

**ISH protocol**

We washed the PS with 95% ethanol for 15 min, and refixed them with 4% paraformaldehyde in 1X PBS (Phosphate-buffered saline) for 15 min at 37°C. The remaining liquid was removed and the slides were air dried at 37°C. We incubated the samples with 0.2N HCl (Hydrochloric acid) for 15 min at 25°C and washed them with 2X SSC (Saline-sodium citrate buffer) (20X SSC is 3 M Na-citrate, pH 7.0) at room temperature for 15 min. We transferred the slides to a covered plastic micro-pipette tip box that we used as hybridization chamber (hybridization box). Each sample was covered with 100 µL of prehybridization solution (ex SSC, 2% blocking reagent, Boehringer Mannheim), 20% formamide (w/v), 0.1% N-lauroylsarcosinate (w/v), 0.02% SDS (sodium dodecyl sulfate) (w/v), and incubated in the hybridization chamber at 37°C for 1 h.

After prehybridization, we added 30 µL of fresh hybridization solution containing the appropriate labeled oligonucleotide at a final concentration of 40 ng/mL, and covered the sample with a coverslip, sealing the edges with nail enamel.

We reincubate the samples at 92°C in the hybridization box, placed in a boiling water bath, for 10 min, and at 37°C for 1 h. After hybridization, we immersed the slides in a staining jar with 6 X SSC solution at room temperature and carefully detached the coverslips. The following washes were applied: one with 6 X SSC at 48°C for 15 min and a final wash with 2 X SCC at room temperature for 15 min.

The detection was accomplished with a Gen detection kit from Boehringer Mannheim following the manufacture’s indications. In general, the color reaction was allowed to proceed up to 6 h, but overnight development yielded good results as well. After stopping the color reaction with TE (Tris/EDTA) (10 mM Tris-HCl, 1 mM EDTA (ethylenediamine tetraacetic acid), pH 7.5), we dehydrated the samples with graded ethanol (from 70% to 99%). The contrast can optionally be increased by eosin counter staining.

We mounted the samples with permount and observed them by light microscopy at 40 X magnification. A positive result was observed as dark-blue or purplish aggregations within the cell nuclei. As positive controls we used in HeLa cells.

**Polymerase chain reaction**

Frozen cervical swabs embedded in PBS were thawed, drained, and the cotton tips discarded. The remaining solution was centrifuged for 30 sec in a microcentrifuge eppendorf, the supernatant discarded, and the cell pellet resuspended in 10 to 20 µL of lysis solution (10 mM Tris-HCL, pH 7.5, 0.1% sodium laurylsarcosinate, 100 g/mL proteinase K). This was followed by incubation for 1 h at 56°C and, finally, the proteinase was inactivated by incubation at 95°C for 20 min. We cleared the lysates with 5 sec centrifuged in a microcentrifuge, and took 1 to 3 µL samples to do the PCR.
The mixture contained: 1 mM of each dNTP; 200 µM of each primer (see Table I); 2 units of Taq DNA polymerase in 1X reaction buffer (10mM Tris HCL, pH 8.3; 50 mM potassium chloride; 1.5 mM MgCl$_2$; 100 g/mL gelatin) in a final volume of 25 µL. An overlay of mineral oil (30 µL) was used to avoid water loss during the thermal cycling. The thermal cycler (Ericomp or Eppendorf) was programmed as follows: one cycle of 5 min at 94°C; a second cycle of 1.5 min at 54°C and 1.5 min at 72°C and 1.5 min at 94°C (repeated 35 times), and a last cycle of 10 min at 72°C. We electrophoresed the PCR products (10 µL) in a 2% agarosa gel, this gel was stained with ethidium bromide and observed under a 330 nm ultraviolet transilluminator. As positive controls we performed the PCR assay on genomic DNA of HPV 6b, 11, 16, and 18 or mixture of them, and in HeLa cells. As negative control we used purified human leucocyte DNA.

Kappa coefficient

It was used to estimate the agreement between PCR and ISH as classifiers of the level of risk of HPV; Cohen JA, (26). The interpretation of the kappa coefficient was done using the classification provided by Landis y col. (27). These values were done using Epi Info 6.04 Dean y col. (28).

RESULTS

Selection and specificity of the oligonucleotide probes

As expected (Fig. 1), the mixture of both oligos (“universal” probe) detected the four viral types, whereas the “malignant” probe only reacted with HPV 16 and 18. No reactions was observed with mixed with cellular DNA.

In situ hybridization

The ISH protocol described in Materials and Methods permitted us to detect HPV risk groups in routine PS preparations. In Fig. 2 we show the results of several ISH experiments. When we used whole genomic probes (included for comparative purposes) on a negative sample (Fig. 2A) and positive one (Fig. 2B), although a difference is observed, we found it difficult to distinguish between samples. On the contrary, the use of digoxigenine-labeled oligoprobes produced a higher contrast. An extreme result is presented in Fig. 2C and D, where oligonucleotides 1 and 2 respectively, were used in HeLa cells. No color reaction was

| TABLE I |
| PRIMERS AND TARGETS USED TO PERFORM THE HPV PCR ASSAY |

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<th>Forward Primers</th>
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<tr>
<td>Target</td>
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observed as expected with oligo 1, whereas a strong reaction embodying the whole cell was observed with oligo 2. Fig. 2E shows a positive result when used oligonucleotide 2 in a PS from a patient suffering an invasive carcinoma, and Fig. 2F shows another positive sample. In both cases, we observed dark purples aggregations within the cell nuclei with additional cytoplasmic color depostions. A negative sample show in Figure 2G shows no color aggregation within cells.

Polymerase chain reaction
Every sample analyzed by ISH was confirmed by PCR assay. This PCR assay simultaneously detects the presence of HPV types: 6, 11, 16 and 18, in such a way that the viral type can be deduced by the size of its products (multiplex PCR). Table I shows the primers used, their respective target and the sizes of the amplified products. Fig. 3 shows the result of doing the multiplex PCR in a mixture of cloned viral genomes, and two samples of human DNA from negative patients. This approach spared us from the cumbersome confirmation of each viral type by DNA hybridization (29).

Fig. 4 shows the PCR results in several cervical swabs, the smaller DNA band, common to all samples (except for lanes 10 and 11), is the positive control of the PCR, and is the product of the amplification of a target within the human ribosomal genes (30). In lanes 2, 3, 4 and 5 we show the result of patients infected with HPV 6b, 11, 16 and 18, respectively. In lane 6, we present another patient infected with HPV 16.

In lane 7, 8, 9 and 12 four negative samples are shown whereas in lane 10 the PCR failed as indicated by the lack of the human ribosomal amplification product. Lane 11 shows the absence amplification when no DNA is added (carry-over-control).

In Table II we summarized the results of ISH and PCR in 156 studied samples, where we obtained a kappa coefficient of 0.945 showing a strong agreement between PCR and ISH, the 95% confidence interval contains values from 0.728 to 1.000.

**DISCUSSION**

The ISH presented here is compatible with the routine cytology/histology labora-
Fig. 2. Microphotographs of the In situ hybridization A and B show two Pap Smear samples hybridized with whole DNA of recombinant virus HPV 16 labelled with digoxigenina. A is negative and B positive. C and D show samples of HeLa cells hybridized; in C with oligonucleotide 1 (bipin probe), and in D with oligonucleotide 2 (malignant probe). E is a PS of a patient with in situ carcinoma, F is a PS of a patient with CIN I both hybridized with oligonucleotide 2. G is a PS of negative patient hybridized with a mixture of oligonucleotide 1 and 2 (universal probe).
tory, and detects HPV genomes in cervical smears with a higher predictive value than the PS. The sensitivity values of ISH are due in part to the amplification power of the microscope, which allows the direct observation of the color hybridization signal within the infected cell. This fact permits establishing a straight association of the viral genome with the epithelial cell origin, an observation which is a very important for the oncologist. The digoxigenine-labeled oligonucleotides used as probes, render a clear and not shown hybridization signal when compared with labeled whole genomes and can discriminate between low risk and high risk viral types in a fast hybridization reaction. Comparing the reaction described here, we could do the screening and the typing of the same HPV by PCR.

Although new anogenital-associated HPV types are discovered each year, HPV 16 (31) and, to a lesser extent, HPV 18 (32) have been found in the majority of the cases of cervical or vulvar malignancy (33-36). In Venezuela, we found (37-40) that nearly 80% of the cases of high grade squamous intraepithelial lesions (SIL) and malignants lesions, contained HPV 16 or 18 DNA sequences.

Although PCR is a very sensitive technique, with a crucial role in diagnostics of human pathogens (41), its high sensitivity is also its major disadvantage. For this rea-
son, extreme sample manipulation precautions must be taken to avoid carry-over contaminations. Nevertheless, using the reaction described here, we could do the screening and the typing of the HPV in a single reaction, reducing the number of samples and the experimental time, as well as the associated costs. In addition, a comparison of the yields of the PCR products with those of the PCR internal control (rDNA), makes it possible to obtain an idea of the extent of the infection (42). For instance, the sample of lane 2, Figure 4, seems to have more copies of the HPV6 genome than the sample of lane 12.

The kappa coefficient between both methods is 0.945, indicating a strong agreement between the ISH and the PCR assays. Our ISH could be compatible with the mass screening required at hospitals and cancer reference centers, and the fact that it can detect high risk viral genomes ahead of any clinical symptom enhances the predictability and diagnostic potential of the traditional PS. In addition, the detection of high risk infections can reduce the costs of diagnosis, since only in these group of patients, the test must be time spaced by shorter periods.

Even when the traditional methods as Papanicolaou cervical smears, it continues being a valuable tool for the screening of VPH, it is important to emphasize that we adapted the system to fulfill the requirements of gynecologist and cytologists, simulating their protocols and simplifying the in situ hybridization assay, when we compared the detection levels reached by in situ hybridization versus a ladder PCR assay.

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