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Molecular detection of human papillomavirus in urine and cytologyc feminine samples

Abstract

Cervical cancer is the second most common in women worldwide; infection with high oncogenic risk Human Papillomavirus (HPV) is the main etiological factor of this malignancy. Viral identification is achieved by sensitive and specific molecular methods such as polymerase chain reaction (PCR) which are used as cervical biopsies or swabs biological material. Because of the problems and complications that imply taking these samples, studies are conducted with other less invasive as urine.

Objectives: the detection and HPV genotyping in endocervical swabs and urine was performed by comparing the results obtained and to evaluate the effectiveness of using urine samples.

Methodology: the genetic material was obtaining using commercial kit Axygen. For viral detection and typing the PCR technique was used.

Results: The positive percentages for HPV the presence were 68.6% in cervical swabs and 62.9% in urine samples, similar values and comparable to previous studies. Likewise, the correlation obtained between viral strains was "good" (0.609), also obtaining high sensitivity and specificity using urine samples 83.3% and 81.8% respectively. These results point to the possibility of developing an effective diagnosis for HPV using urine samples, because it reduces the intervention of trained personnel, cost and discomfort for patients.

Keywords: HPV, urine, cytologyc, endocervival, UC

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Introduction

Cervical cancer is the second most common in women worldwide, it is estimated that it causes around 500,000 deaths a year worldwide, with an incidence close to 40 per 100,000 women in developing countries.[1] In Venezuela, in 2006, it was the second cause of cancer death in women, with 2,141 cases and 4.56% of the total number of female deaths that year.[2] Official data from the Ministry of the Popular Power for Health, point out that for the year 2007 1,571 women died from cervical cancer with a morbidity of 3,700 cases, being the first cause of death by cancer in the female population, overcoming the cases of death for breast cancer.[3] Studies on its causes and associated pathogenesis have increased rapidly. Persistent infection with high-risk HPV has been defined as one of the main causes of most cases. Viral DNA is found in more than 99% of squamous cell carcinoma of the cervix (CCU) biopsies, of which 70% corresponds to infections with high oncogenic risk genotypes.[4] HPV comprises a large group of more than 100 viral types belonging to the family of Papillomaviridae.[5] Around 40 genotypes are transmitted via sexual contact or by direct contact in humans that infect the skin and the genital and oral mucosa, producing one of the most common sexually transmitted diseases in the world.[6]

In the last 20 years, more than 100 genotypes have been characterized, which are discriminated according to the similarity of their DNA using probes of viral HPV genotypes known by means of molecular hybridization techniques. Based on its oncogenic capacity, the different HPVs have been grouped into types of high oncogenic risk, such as 16,18,31,33,35,39,45,51,52,56,58,59,66 and 68 associated with invasive cancer and squamous intraepithelial lesions of low and high grade (LIEBG and LIEAG); and types of low oncogenic risk such as 6,11,42,43 and 44 mainly associated with genital warts, LIEBG and recurrent respiratory papillomatosis.[7] Each genotype of HPV acts as an independent infection, with different degrees of oncogenic risk. New research has led to better prevention and clinical management strategies, including viral detection tests and improved vaccines. The new models oriented in the relationship between HPV and cervical carcinogenesis should promote a strong base in conjunction with the oldest morphological models based on cytology and histology, since the advances in molecular biology are available. Essential tool for the early detection of infection by the virus.[8]

Given the association between CCU and HPV, all studies are important to improve or facilitate viral detection as well as to evaluate the type of biological sample used. The objective of the present work is then to establish the viral detection in urine samples in order to simplify the sample taking aiming at the possibility of developing an effective diagnosis as it would provide a means of viral detection, which generates less costs and discomfort for the patient and in which the intervention of trained personnel and specific equipment is not indispensable for taking the sample.[6]

Methodology

Biological sample

The investigation was carried out with female patients who spontaneously attended the consultation of the Dermatology Service of Sexually Transmitted Infections (ITS) of the University Hospital of Caracas (HUC), to which a survey was conducted. An informed consent was signed to express approval to participate voluntarily in the study; both documents were previously approved by the Bioethics Committee of the HUC. The patients were recruited during the year 2013. The samples to be processed corresponded to swabs and urine from 35 patients with a clinical diagnosis of HPV infection. We also

evaluated 18 patients without apparent lesions on clinical examination, with an age superior to 14 years; the minors of this age counted on the approval of their legal representative, and to have initiated their sexual life previous to the study. The cytological and urine samples were transported and stored at 4°C and -20°C respectively. Exclusion criteria for the study are pregnant patients, patients with compromised immune systems or HIV positive and patients who did not sign informed consent.

Extraction of genetic material

The extraction of the genetic material was carried out using the commercial kit "DNA-kit Axygen Biosciences" following the specifications of the commercial house, with a preliminary treatment according to the sample used

Hyssop

It began with the detachment of the exfoliated cells vortexing each shows for 5 min at maximum speed. The cell suspension was transferred to a 2mL microcentrifuge tube and processed with the kit "DNA-Kit Axygen Biosciences" according to the indications of the commercial house.

Urine sample

The urine sample was centrifuged at 20,000g for 1 hour to sediment the cells. The pellet was washed with 400µl PBS 1X and centrifuged for 15 minutes at 20,000g. The pellet was resuspended in 200µl of 1X PBS or saline solution and transferred to an Eppendorf, to carry out the extraction of nucleic acids using the case kit to the DNA-Kit Axygen Biosciences, following the specifications of the commercial house.

HPV detection by conventional PCR with primers MY09/11

The detection of the viral genome was carried out by PCR with generic primers MY09 and MY11, which recognize the L1 consensus region of the viral genome to obtain an amplification product of 450bp.[9,10] The PC04/GH20 primers were used to simultaneously amplify a fragment of the human β-globin gene as internal control of DNA integrity and quality and absence of PCR inhibitors, with which a product of 268bp is obtained.[11] A negative control (reaction mixture+distilled water) was also included. Approximately 1µg of the extracted DNA was added to the reaction mixture [0.4µl dNTP's (100mM); 0.2µl of each primer (100mM); 6µl Taq buffer 10X; 4µl $MgCl_2$ (50mM); 0.5µl (2.5U) of Taq DNA polymerase (Invitrogen); H_2O free of nucleases up to a final volume of 40µl] and amplified in an eppendorf thermocycler [Cycles: 4min at 94°C, 40X (15sec at 94°C, 30sec at 55°C, 45sec at 72°C) and a final extension of 7min at 72°C].

HPV typing

The positive samples in the viral detection with the MY09/11 primers were typed by means of multiple PCR, using the primers described by Sotlar et al.[12] This multiple PCR allows the following viral types to be identified: 6/11 (334bp) of low oncogenic risk, 16 (457bp), 18 (322bp) and 33 (398bp) of high oncogenic risk. For this, 3µL of the DNA of each sample was added to the reaction mixture, which consisted of [0.4µl of dNTP's (100mM), 1.6µl of the initiator cocktail (100mM 0, 2µl of each primer), 0.5µl (2.5U) of Taq DNA polymerase (Invitrogen), 4µl of $MgCl_2$ (50mM), 6.5µl of Taq Buffer (10X) and 27µl of H_2O nuclease-free up to a final volume of 40µl] and amplified in an eppendorf thermocycler [Cycles: 4 min at 94°C, 35X (30 sec at 94°C, 45 sec at 56°C, 45sec at 72°C) and an extension end of 4min at 72°C].

Electrophoresis

The products of the amplifications were visualized by electrophoresis in 2% agarose gels, TBE 1X buffer (Invitrogen), stained with SYBR Safe 1X (Invitrogen), at 100V for approximately 45min. They were then exposed to UV light for photographic recording, on a ChemiDoc™ XRS+(Bio-Rad) viewing camera.

Statistics tests

Sensitivity and specificity values of viral detection were determined when urine samples were used. To do this, statistical tests were used (SPSS 2.0program) and the equations described in Fernández & Díaz.[13] The results obtained in the urine samples were compared with the end cervical swabs to evaluate sensitivity and concordance of the viral detection. For the evaluation of sensitivity and concordance, statistical tests were used using the SPSS 2.0program.

Results

We studied 53 women, of these, 35 corresponded to patients with a diagnosis of HPV infection through clinical gynaecological examination; additionally, samples were taken to 18 women without visible lesions to the clinical examination, which allowed to evaluate the usefulness of the urine samples in the detection of latent or subclinical infections. The average age of the patients was 23.8±11.41 years, being the 20 years of age the most observed age. The smoking activity was present in 37.2% (13/35) and the consumption of alcohol in 74.3% (26/35) of the patients. The age of menarche showed an average of 12.20±1.66 years, with 12 years being the most value with 37.1% (13/35) of the patients. The number of sexual partners had an average value of 2.52±1.37, with 2 couples being the most frequent value, in a period of 6 months to a year. Regarding the marital status of the infected patients, it was found that 91.4% (32/35) were single, and 8.6% (3/35) were married; in this last category, a stable partner is assumed. In the population of women studied with a clinical diagnosis of HPV infection, the overall prevalence of infection by this virus was 68.6% (24/35) in cervical swab samples and 62.9% (22/35) in urine samples. (Table 1) Among patients positive for HPV in cervical swabs, 45.8% (11/24) presented HPV of low oncogenic risk (genotypes 6 and/or 11), 4.1% (1/24) of high risk oncogenic (genotype 16) and 50% (12/24) could not be typified by the methodology used (Table 1). Of the patients positive for HPV in urine samples 36.36% (8/22) presented HPV of low oncogenic risk (genotypes 6 and/or 11), 9.09% (2/22) of high risk oncogenic genotype 16 and 4.54% (1/22) of high risk for genotypes 18 and 33, 9.09% (2/22) mixed infection (HPV of low and high risk oncogenic 6 and/or 11 and 16) and 36.36 % (8/22) could not be typified by the methodology used (Table 1).

In addition, a positivity correspondence was obtained between the results obtained in the detection process by means of the two types of samples of 57% (20/35). From this percentage, positive patients in whom the HPV genotype coincided were determined, obtaining a correspondence of 40% (8/20) for the identified genotypes in which genotypes 6 and / or 11 of low oncogenic risk are included and 16 of high oncogenic risk. Of the group of patients who did not present lesions suggestive of HPV infection to the clinical examination, the general prevalence of infection by this virus in both types of samples

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was 33.3% (6/18), which highlights the usefulness of molecular techniques in the detection of subclinical infections and the importance of using procedures that allow the direct identification of the viral genome. Of this group, in those samples corresponding to cervical swabs, 16.6% (3/6) presented HPV of low oncogenic risk, 11% (2/6) of high oncogenic risk and 5.5% (1/6) could not be typified by the methodology used. In the urine samples it was found that 5.6% (1/6) had HPV of low oncogenic risk and 27.8% (5/6) were not typified by the methodology used. With the Chi-squared statistic, a low degree of independence was obtained (p=0.01), which allows to establish similarity between both types of samples and a good concordance of the results (k=0.609), by means of viral detection and typing. Through the conventional and multiple PCR technique.

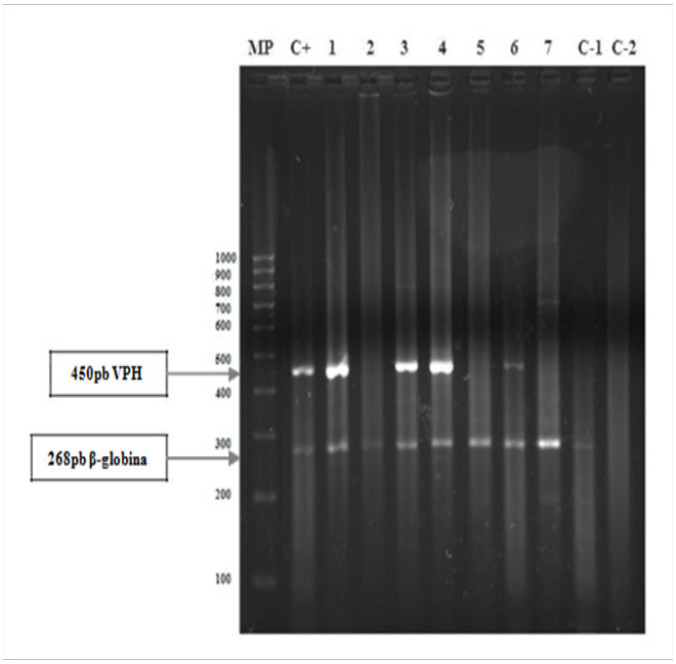

Figure 1 Visualization of HPV detection by agarose gel electrophoresis to 2%; MP: Molecular weight marker (100 bp Invitrogen); C+: Positive control (indicator at 450bp) and internal control of DNA integrity (β-globin gene at 268bp); 1,3,4 ,6: Samples of HPV positive patients; 2,5 and 7: Patient samples HPV negative C-1: Negative control with DNA from a healthy patient (the 268bp band corresponds to the β-globin gene); C-2: Negative control (only reaction mixture).

Table 1 Percentage distribution, for the detection and typing of HPV in samples of cervical swabs and urine samples. n=35 patients

HPV infection frequency		
	Cervical swabs	**Urine samples**
Positive	68.80%	62.90%
Negatives	31.40%	37%
Genotypes		
11-Jun	45.80%	36.36%
16	4.20%	9.09%
18	-	4.54%
33	-	4.54%
6/11 and 16	-	9.09%
NT	fifty%	36.36%

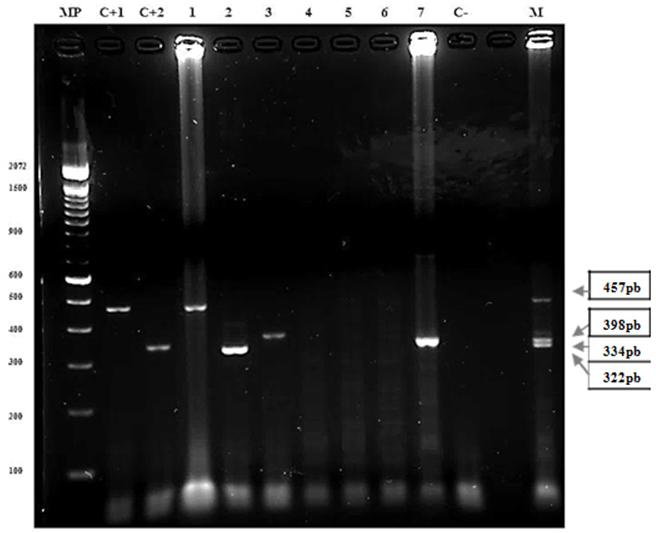

Figure 2 Visualization of HPV typing by agarose gel electrophoresis to 2%; MP: Molecular weight marker (100bp Invitrogen); C+1: Positive control of HPV type 16 (indicator at 457bp); C+2: Positive control of HPV type 6 and/or 11 (indicator at 334bp); 1: Patient sample of positive VPH type 16; 2: Sample of positive HPV patient type 18; 3: Sample of patient VPH positive type 33; 4,5,6: Non-typifiable HPV patient samples by this methodology; 7: Sample of positive HPV patient type 6 and/or 11 (334 bp); C-: Negative control; M: Positive control with the mixture of HPV genotypes type 18 (322bp), 6 and/or 11 (334bp), 33 (398bp) and 16 (457bp).

Discussion

Although it has taken around 20 years to establish the causal association between cervical cancer and persistent HPV infection, at present the natural history of infection with this virus and this malignancy is widely known and, therefore, tests have been developed of high and low risk HPV DNA detection to improve efficiency, accuracy and effectiveness in the timely detection of cervical cancer, as a secondary prevention strategy.[14] The average age of the study population was 23.8±11.41 years and presented several of the risk factors mentioned as alcohol consumption, tobacco, contraceptive use, early sexarquia, more than one sexual partner in a period of 6 months to a year. These characteristics or conditions are considered by authors as Walboormers et al.,[15] Bosch et al.,[16] Moreno et al.,[17] & Hildesheim et al.,[18] cofactors in the risk to develop CCU.

Additionally, the presence of HPV DNA was detected in 68.8% of the cervical swabs and 62.9% of the urine samples, with a positive correspondence of 57%; The discrepancies in the positivity between the samples of the same patient can be a consequence of the type of sample evaluated, the distribution or location of the virus and the viral load at the moment of the capture.[19] This agreement was lower than that obtained by Stanczuk et al.[20] of 79%, who worked with 43 patients who had cervical swabs and urine samples,[20] and Alameda et al.,[21] of 80% when working with cervical swabs and urine samples from 50 patients with a clinical diagnosis of high and low grade LI.[21] The difference between these values of agreement and that reported in the present study can be attributed to differences in the characteristics of the populations studied, the degree of the lesions and/or the methodology used in each study. Regarding the percentage of detection, when compared with other studies, Yerena et al.,[22] evaluated 123 female patients and reported a 12.2% positive for

the virus, because these patients presented normal cytology results, in addition they establish comparisons with the results obtained by Torrella et al. Cited by Yerena et al.,[22] 17% and Carrillo et al.,[23] with 23.1%, values below that obtained in the present study.[23]

In relation to the results of different studies on HPV detection with urine samples, there is great variability, including negative values. When comparing the percentage of HPV detection with urine samples obtained in this study with those of other studies, such as that of Tanzi et al.,[24] is considered as a lower value, since the researchers reported a detection rate of 64.5%.[24] On the other hand, Alameda et al.,[21] evaluated 50 urine samples from women from Barcelona, Spain, with an average age of 36 years, obtained 22% (11/50) of positivity for HPV DNA by PCR with primers MY09/MY11,[21] from likewise, Sellors et al.,[25] evaluated 200 urine samples from women in Hamilton, Canada, with an average age of 31.5 years, referred for abnormalities in cervical cytology, finding positivity of 35% (69/200) for the DNA of the virus.[25] Within the positive samples in the viral detection, four different genotypes were identified; in cervical swab samples genotypes 6 and/or 11 of low oncogenic risk were obtained in 45.8%, 16 of high oncogenic risk in 4.2% and 50% could not be typified by this methodology. In the typification of the urine samples genotypes 6 and / or 11 of low oncogenic risk were identified in 36.36%, 16 of high oncogenic risk in 9.09%, 18 of high oncogenic risk in 4.54%, 33 of high oncogenic risk in 4.54%, mixed infections with types 6 and/or 11 and 16 of high and low oncogenic risk in 9.09% and 36.36% of the samples was not typifiable by the methodology used. Regarding the viral types detected in Venezuela in previous studies, using different methodologies, such as Correnti et al.,[26] and others from different countries such as Muñoz et al.,[27] Suarez et al.,[28] Ghaffari et al.,[29] & Aedo et al.,[30] who made the identification of high-risk HPV oncogenic, in contrast to the results of the present work where it was detected mainly HPV of low oncogenic risk. This could be due to differences in the methodologies used in the detection and typing, as well as to the geographical area and the type of injuries of the population studied in each case. These results are similar to those reported by Scucces & Paneccasio[31] who obtained 25% detection of low oncogenic risk virus.[31] It should be noted that it should be taken into account that there was a percentage of patients who were positive for high-risk oncogenic HPV, so a more active surveillance is recommended in these cases specifically.[32] To compare viral detection and typing with cervical swabs and urine samples, the determination of sensitivity and specificity was carried out, taking into account the positive and negative predictive values with respect to the samples used, according to Fernández & Díaz.[13] A sensitivity of 83.3%, a specificity of 81.8%, a positive predictive value (PPV) of 90.9% and a negative predictive value (NPV) of 69.2% were recorded. This means that there is a high probability of effectively determining the presence or absence of HPV DNA in urine samples, since values above 80% were obtained. These are similar to those reported by Tanzi et al.,[24] who obtained a sensitivity of 98.6%, a specificity of 97.4% in the detection of HPV DNA in urine in a job where they also used cervical swabs.[24]

The difference in the percentage of HPV detection using urine samples is slightly lower than that obtained with cervical swabs. However, according to the statistics applied, a good concordance was obtained. This difference could be eliminated by applying methodological variations such as; using a larger volume in the collection of urine. In this regard, the work of Vorsters et al.,[19] consists of a compilation of 41 studies describing volumes of urine collection ranging from 400-600mL for the approximate collection of 200µl of desquamated cells from the urethral canal in a compact sediment.[19] The authors point out that by increasing the volume of urine for the study, the percentages of viral detection in this type of sample could be improved and, therefore, a higher estimate of dependency and concordance in the results would be achieved. In addition to the studied sample composed of female patients with a history of HPV, a sample was also evaluated consisting of women without clinical and/or cytological findings suggestive of HPV infection and with no history of it, finding 33.3% positivity for the patient. DNA of the virus in both urine samples and cervical swabs, detecting the existence of possible latent infections in patients who are healthy on clinical examination. Works as the compilation of Vorsters et al.,[19] several of the authors point out the importance of incorporating the detection of viral DNA in research programs along with conventional cytology, considering that this would contribute to the detection of subclinical infections and this, in turn, would increase the efficiency of programs of control and prevention, besides contributing with the correct surveillance of patients and the success of the treatments.[19]

The observed results are encouraging since there is similarity between those obtained with each type of sample, pointing to the possibility of using samples of urine as an alternative or supportive method for the detection of viral infection, in women with little gynaecological care or with cultural and religious limitations, as well as in geographies of difficult access. In this sense, the use of this type of sample could become a useful variant in the detection of HPV infection and the screening of CCU, since it has the following advantages: it does not need the intervention of trained personnel for its collection; it is a Painless procedure, low cost for the patient and non-invasive. These investigations will allow evaluating the potential of the use of urine samples in the detection of HPV, as well as the possibility of its use in health programs and screening of cervical cancer (CCU). It is important to mention that in Venezuela there are no studies on the use of urine samples for the detection of HPV, which may be the reference in future research.

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Conflict of interest

The author declares there is no conflict of interest.

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