Prevalence of Human Papillomavirus (HPV) Genotypes and Multiple Infections in Routine Cervical Cancer Screening in a Spanish Regional Population

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Abstract

**Background:** Infection with human papillomavirus (HPV) has been identified as the primary cause of cervical cancer. Persistent infection with high-risk-HPV is the required factor for the development of cervical cancer.

**Objective:** The aim of this study was to know the prevalence and distribution of cervical HPV infection in women attending routine cervical cancer screening.

**Study design:** Liquid-based cytology (LBC) of cervical specimens was processed for HPV testing by INNO-LIPA HPV genotyping Extra Reverse Hybridization Line Probe Assay kit.

**Results:** The cytological results of 115 LBCs were: 11(9.6%) negative for intraepithelial lesion or malignancy (NILM), 32(27.8%) atypical squamous cells of undetermined significance (ASC-US), 62(53.9%) low-grade squamous intraepithelial lesion (LSIL) and 10(8.7%) high-grade squamous intraepithelial lesion (HSIL). No cases of cervical cancer were detected. The mean age was 33.78 years ± 10.75 years (range 17-62 years). The overall prevalence DNA HPV detection was 81.7% (94/115), with a large proportion of multiple HPV infection (55.0%). HPV16 was the most common type detected (8.7%) followed by HPV51 (7.8%), HPV52 (5.2%), and HPV66 (2.6%). We found a high proportion of HPV positive cases in all cytological categories, including NILM cases.

**Conclusions:** These results indicate that prevalence of HPV infection in women attending routine cervical cancer screening is high. Our results confirm the importance of identifying the types of HPV that affect this community to design more effective prevention strategies and thus contribute to the fight against cervical cancer and its precursor lesions.

**Keywords:** Liquid-based cytology; Screening cervical cancer; Prevalence; HPV; Multiple HPV infection.

Background

Cervical cancer is the second most common cancer in women worldwide and the most common cause of mortality in underdeveloped and developing countries [1,2]. Persistent infection with certain oncogenic high-risk (HR) types of Human Papillomavirus (HPV) is the required factor for the development of invasive cervical cancer and precursor lesions [3-6].

Overall, the prevalence and distribution of HPV infection in cervical lesions has been determined in many geographical regions of the world, this can vary depending on several factors including epidemiological differences in the populations studied and the methodology used for molecular detection and typing of HPV DNA [7]. Over 120 types of HPV have been classified as either low (LR-HPV) or high risk (HR-HPV), according to their oncogenic potential [8,9]. Within the high-risk genotypes, HPV types 16 and HPV 18 are most often associated with cancer and squamous intraepithelial lesion [5,6,10,11,12]. Nevertheless, the risk of neoplasia for other types of HPV as well as for multiple HPV infection has not yet been established.

Because current strategies for the prevention of cervical cancer and their precancerous lesions are based on the HPV genotyping [13] and prophylactic vaccines [14-16], it seems necessary to determine the types of HPV most commonly associated with malignant cell transformation in different geographical areas in order to establish effective preventive measures according to the epidemiology of the population. This study was to know the prevalence and distribution of cervical HPV infection in women attending routine cervical cancer screening in the Valencia region (Spain).

**Study design**

Liquid-based cytology (LBC) of cervical specimens was collected during routine screening visits, between June 20, 2011 and September 9, 2011 in the Clinical Hospital of Valencia, Spain. LBC samples were processed using the Thin Prep® 5000 processor (Madrid, Spain). For HPV detection and genotyping, we used the residual cervical sample material available from the same Thin Prep Pap test vial previously used for cytological screening. DNA was extracted from the residual materials of LBC by QIAamp® DNA mini kit (IZASA, Valencia, Spain), according to the manufacturer’s instructions. HPV testing was performed by INNO-Lipa HPV genotyping Extra Reverse Hybridization Line Probe Assay kit (Innogenetics®, Barcelona, Spain), according to the manufacturer's instructions. Briefly, HPV detection and genotyping was based on PCR amplification of a 65pb fragment, within the L1 region of the HPV genome, using broad-spectrum SPF10 biotinylated primers. PCR was performed in a final
reaction volume of 50μl containing 40 μl of PCR master mix and 10μl of the extracted DNA. The amplification programme took 9 min at 94°C followed by 94°C for 30 sec, 52°C for 45 sec and 72°C for 45 sec for a total of 40 cycles, with a final extension of 10 min at 72°C. For hybridization, we used the AutoBlot 3000H INNO-LiPA HPV genotyping Extra procedure (Innogenetics, Barcelona, Spain), according to manufacturer’s instructions. The LI-RAS® (Innogenetics, Barcelona, Spain) for LiPA HPV software was designed to assist with genotyping extra results. The samples for which the obtained line pattern could not be assigned to any genotype pattern or which had no type-specific lines, but had at least one HPV control line positive were considered HPV-positive but undetermined (HPVX).

Statistical analysis

Statistical analysis was performed with the SPSS v17.0 statistical package. A p value of 0.05 or less was considered statistically significant.

Results

A total of 115 liquid-based cervical cytology were included in this study: 11(9.6%) were negative for intraepithelial lesion or malignancy (NILM), 32(27.8%) were atypical squamous cells of undetermined significance (ASC-US), 62(53.9%) were low-grade squamous intraepithelial lesions (LSIL), and 10(8.7%) high-grade squamous intraepithelial lesions (HSIL). No case of cervical cancer was diagnosed. The mean age was 33.8 years (standard deviation, 10.75) and age range 17-62 years.

The cases were divided into two age groups <40 and ≥40 years. The major proportion of cases studied (70.2%) belonged to patients <40 years, while 29.8% were ≥40 years. 90.4% of cases (103/115) had abnormal Pap smears (ASC-US, LSIL and HSIL) distributed irrespective of age (p= 0.078), however, 73/103 (64.0%) were <40 years.

94 out of 115 cervical specimens were HPV DNA positive (81.7%). The highest proportion of positive HPV cases (60.5%) corresponded to patients younger than 40 years. 21.1% of patients ≥ 40 years also had viral infection with statistically significant differences (p=0.048).

Among the HPV positive specimens, 45.0% (42/94) were identified as single HPV types and 55.0% with multiple HPV types, with statistically significant differences (p<0.001). At least one high-risk HPV genotype was detected in the samples with HPV co-infection. The four most common single types among positive cases were: 10 HPV16 (8.7%), 9 HPV51 (7.8%), 6 HPV52 (5.2%), and 3 HPV66 (2.6%). Also 10 undetermined HPV (HPVX) were positive cases (8.7%). HPV16 was present in 20.9% of HPV-positive samples, including single and multiple HPV infection (Table 1). The most frequent viral types in this study were HR-HPV. Few LR-HPV types were found in the oncogenic cases studied. The most frequent virus type was HPV16, both as single or multiple. HPV18 was rare. Types HPV31 and HPV33 were frequent in multiple infections cases; Types HPV-51,-52 and -66 were very common in single and multiple infections, combined with other HR-HPV types. HPVX was often present in the form of single infection and was present in 9 cases of coinfection (Table 1).
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1). HPV39 was frequently found in combination with other HPVs. HPV11 was the most common LR-HPV type.

28.1% of cases in the <40 years group were infected by one HPV type, as well as 8.0% of women ≥ 40 years. Multiple infection was detected in 32.5% (37/115) women <40 years and decreased with age, 12.3% (14/34), without statistically significant differences (p=0.133).

We found a high proportion of HPV positive cases in all cytological categories, including NILM cases (Table 2). The distribution of HPV positivity between cytological categories is shown in Figure 1. Thirty-eight cases of LSIL (33.0%) had multiple HPV infection, followed by 8 ASC-US (7.0%), while only 6.1% of HSIL were infected. Multiple infection was more common in LSIL and ASC-US, while single HPV infection was more frequent in HSIL.

Discussion

At present, it is very important to know the distribution of other types of HPV that enhance or decrease the risk of developing cervical neoplasia. The distribution of HPV types in cervical lesions is crucial to the design of second-generation prophylactic vaccines effective for specific populations.

In Spain, crude prevalence rates for HPV infection in small, specific populations or in limited geographical areas have been previously reported to range from 3% to 17% [17-20]. In a study to provide specific information for Spain in 1,043 histologically confirmed invasive cervical cancer cases from six regions, 904 of these cases (89.1%) were HPV DNA positive [5]. In this study, a high prevalence of HPV infection (81.7%) as well as a high diversity of oncogenic HPV types was observed. This proportion is equivalent to that found in abnormal Pap tests from a population-based screening program [21], in HIV-positive Brazilian women [22] and in a study of worldwide HPV genotype distribution in cervical cancer [12]. While HPV infection was detected in 98.4% of women with histologically confirmed CIN2 or higher [23]. The presence of cervical lesions in the populations studied could be the reason for the similarity in the prevalence of HPV infection.

The high prevalence of HPV infection is common in young women [6,24,25]. In all geographical regions, HPV prevalence was highest in women younger than 35 years of age, decreasing in older women [26]. Regarding age distribution, 69% of all HPV infections were found in women aged 20-29 years [27,28].

In this study, the prevalence of HPV (81.7%) of women living in this Spanish region was higher than that observed in Spanish data published previously among women aged 18-65 years (14.3%) and women aged 18-25 years (28.8%) [6]. Furthermore, we found highest prevalence of HPV (60.5%) in patients <40 years, even higher than that reported in other studies carried out in Spanish women, probably because it encompasses a different age group [6]. In previous studies conducted in this same context, HPV infection was positive in 90% of the patients with abnormal cytology and a mean age of 40 years [29], and in 43.3% in patients with mean age of 40.9 years of the routine screening [30].

The distribution of HPV genotypes and prevalence estimates are dependent on population characteristics such as age, sexual behavior, severity of cervical disease, and the geographic location of the patients as well as of the method(s) of detection and indicate that suboptimal analytical sensitivity for one or more of the less common HR-HPV genotypes could lead to impaired clinical sensitivity [6,10,31-33].

In this study we found a high prevalence of HPV infection in cases with normal cytological findings (NILM). This high prevalence suggests that it belongs to an epidemiologically relevant population, which should be monitored in order to prevent the occurrence of HSIL or cancer. The study also allows the monitoring of the evolution of the viral infection, and the role of synergism between HPVs involved in the development of cervical neoplasia. Overall HPV prevalence in 157,879 women with normal cervical cytology was estimated to be 10.4% [26]. In Spain, about 13% of NILM cases are HPV positive [6]. The present findings confirm the very high prevalence of HPV infection in patients with normal cytology of this area of Spain [30].

The distribution of HPV genotypes in this study was consistent with that reported in studies previously [20,29,30,34]. HPV16 has been consistently identified as the most frequent HR-HPV. Other HR-HPV types have also been frequently detected. The more common HR-HPV genotypes prevalent in the CLEOPATRE study [4] were HPV-16, -51, -52 and -66, which were also present in this study. HPV16 was detected in 16.9% of samples of the

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Cytological findings and distribution of detection HPV results.

<table>
<thead>
<tr>
<th>Cytological findings</th>
<th>HPV</th>
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<tbody>
<tr>
<td></td>
<td>Negative</td>
</tr>
<tr>
<td>NILM (n=11)</td>
<td>2 (18.0%)</td>
</tr>
<tr>
<td>ASC-US (n=32)</td>
<td>14 (44.0%)</td>
</tr>
<tr>
<td>LSIL (n=62)</td>
<td>4 (6.5%)</td>
</tr>
<tr>
<td>HSIL (n=10)</td>
<td>1 (10.0%)</td>
</tr>
<tr>
<td>Total (n=115)</td>
<td>21 (18.3%)</td>
</tr>
</tbody>
</table>


Table 2: Distribution of HPV detection results according to cytological findings.
While the role for some HPV types, such as HPV16 and HPV18 in carcinogenesis cervical is well established, the role of other HPV types is less clear. In this study, HPV-51 (7.8%), -52 (5.2%) and -66 (2.6%) were detected. These viral types appear to be common in the Spanish population [6,18,35-38] and these HPV specific-genotypes were also detected in studies around the world. The proportions detected in these studies are diverse, being as high as 17.5%, 12.0%, and 8.1% respectively [35], while in other study [38] found similar rates to those of our study: 8.0%, 3.8%, and 7.1%, respectively. Very low proportions have also been detected, such as 1.6%, 1.8%, and 1.2%, respectively [6], and HPV-51 (0.75%), HPV-52 (2.25%) and HPV-66 (0.19%) [10]. Finally, considering the study which was estimated the prevalence of HR-HPV in Latin American immigrants in Spain finding a high prevalence of HPV51 (13.0%) and HPV66 (27.0%) [18], and that Valencia is a cosmopolitan city, with a large population of immigrants, it is also important to take these findings into account when designing future preventive strategies [10]. These types of HPV are of interest after Fife et al. [39] suggested that those viruses might cooperate with HPH16 in the development of cancer. Therefore, the HPV-51, -52, and -66 genotypes should be considered in future research to determine the true prevalence and contribution in the progress of HSIL to cervical cancer [40,41].

Interestingly, in this study a high prevalence of undetermined HPV (HPV X) was found (8.7%). Other studies in which the LIPA method was used for typing of HPV have found a prevalence of 8.3% of HPV in women with histopathologically confirmed cervical lesions [42], in 2.2% of women diagnosed with cervical lesions [43] and 1.0% in cervical cancer cases [12], Rabelo-Santos et al. [44] who used the dot blot hybridization method found 6% of HPVX, and Castellsague et al. [6], who used exactly the same method as in this study, found 3.0% of HPVX. Nevertheless, the HPVX prevalence established by Goncalves et al. [22] in comparison with our and other studies was very high (18.7%). Considering the high prevalence of HPVX in this environment, it is recommended to separate these cases and submit them to sequencing for correct identification, evaluation of the degree of oncogenicity, and consideration for prophylactic vaccines.

Ccoinfection with multiple HPV types is common in younger women with cervical lesions and a poor immune response. Multiple HPV infections could be a clinically important finding, although the significance of infection with multiple HPV types in cervical carcinogenesis process is not known [45-49]. Multiple HPV infections are common in women with ASC-US and LSIL. The ALTS study 2000 [50] found that 58.9% of the subjects with LSIL had between two and six different HPV types. Prevalence of HPV infection depends on the age of the study population [51]. In women aged below 30 years, there was a predominance of multiple infections and ASC-US/LSIL was associated with multiple infection and HSIL with single infections [36].

Multiple HPV infections decrease with severity of lesion, being less frequent in HSIL [34], a fact that coincides with the present findings. However, in a study of 298 women with abnormal cervical cytology in Kuwait [27], reported that as the severity of the cytological diagnosis increased the proportion of single infections decreased, while the proportion of multiple infections increased, and Fife et al. [39] found 85.3% of multiple HPV infections in dysplasia and 61.9% in ASC-US, these last concluding that their data support a possible role for multiple HPV types in the development or progression of cervical dysplasia. This is an issue that requires further investigation.

In this study, 55.0% of cases were positive for multiple HPV infections being more frequent in cases of LSIL [52,53]. The prevalence of multiple HPV infection has been reported in a range that varies from 54.0% to 78.9% [21,46,54-56]. Our results fall within this range. This prevalence of multiple HPV infection is higher than found in other studies conducted in Spain in which the values range between 4.7% and 52.1% [6,29,30,34,36,38,57]. Furthermore, in a study of patients with abnormal cytology obtained a higher value (58.1%) [35] than found in this study. The variations observed could be explained by the geographical diversity of the population studied [58] and the different molecular methodologies used. Most of these studies, including ours, used highly sensitive molecular technology designed to reach a large number of specific HPV genotypes. The incorporation of highly sensitive and specific molecular methodology in screening for cervical cancer and typing of HPV could explain why-increasingly more cases of coinfection with several types of HPV are detected [10,22,59].

In conclusion, the infection with multiple HPV types is frequent in women attending routine cervical cancer screening. We found a high proportion of HPV positive cases, even in cytological NILM cases. Multiple HPV infection is very common in patients with normal and abnormal Pap smears. There is a diversity of HPV types, and HPV16 remains the most common type both in patients with normal and abnormal cytology. The true prevalence of HPV types-51, -52 and -66, as well as the identity of HPVX remains unknown, therefore it seems necessary to pursue studies in order to establish their oncogenicity. Our results confirm the importance of recognizing the types of HPV in women of Valencia to design more effective prevention strategies and thus contribute to improving the fight against cervical cancer and its precursor lesions.

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Contributors

AFI and ALB participated in the study design and interpretation of the results. MTM participated in data collection and analysis, interpretation of the results, and writing the report. All authors provided approval of the final draft of the report.
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