ORIGINAL ARTICLE

Frequency of human papillomavirus infection and genotype distribution among women with known cytological diagnosis in a Southern Italian region

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Key words

HPV type distribution • Pap test • Southern Italy

Summary

Introduction. *In the Puglia region (South Italy) about 200 new hospitalizations for cervical cancer are registered every year. The study investigated the frequency of Human Papillomavirus (HPV) infection and the genotype distribution of HPV in a sample of women with known cytology attending the outpatient clinics of four Gynecological Departments of the University of Bari over a four-year period (2005-2008).*
Methods. *Cervical samples from 1,168 women were analyzed for the presence of HPV-DNA through Polymerase Chain Reaction (PCR) in L1 region and reverse hybridization. The cytological results were associated with HPV positivity and type-specific prevalence.*
Results. *Overall, HPV infection was found in 355 (30.4%) women. HPV-DNA was found in 34.4% of women with a cytological diagnosis of ASCUS, in 46.8% of women with Low-grade Squamous Intraepithelial Lesion (LSIL) and in 87.0% of women with High-grade Squamous Intraepithelial Lesion (HSIL)/carcinoma. Also 16.0% of women with normal Pap smear were found to be HPV-DNA positive. The most common HPV genotype was type 16 found in 27.3% of positives, followed by type 53 (11.5%), type 66 (9.2%) and type 31 (9.0%). HPV genotype 18 was found in 6.4% of positives. Types 16 or 18 were detected in about 34% (120/355) of all infected women, in about 33% of LSIL and in 60% of HSIL/carcinoma HPV-positive women. Among low risk (LR) genotypes, type 61 was found in 10.7% of HPV positive women, type 62 in 8.4%, type 42 in 8.1% and type CP6108 in 7.8%.*
Discussion and conclusions. *The findings of the study give evidence that HPV infection is frequent in the studied cohort of women. The most widespread genotypes found were 16 and 53. These data may represent a benchmark for future evaluation after the recent introduction of vaccination against HPV in 12-year-old girls.*

Introduction

Cervical cancer is the second most common cancer in women worldwide for incidence and mortality [1] and also the leading cancer in developing countries where more than 80% of the cases and related deaths occur [2]. In Italy, about 3,500 new cases and 1,500 deaths are reported every year [3].
Different Human Papillomavirus (HPV) strains frequently infect the genital tract and both epidemiological and experimental evidence show that some carcinogenic strains are associated with cervical cancer [4, 5]. Genital HPV types have been subdivided into high-risk, probable high-risk and low- risk genotypes according to their association with invasive cervical cancer [5, 6]. Types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82 should be considered carcinogenic or high-risk (HR-HPV) whereas types 26, 53, and 66 should be considered probable high-risk (PHR-HPV) [5]. Among high-risk genotypes, HPV-16 is associated with about 60% of the cases of cervical cancer, HPV-18 with 10%, HPV-31 and HPV-45 with 4% each, whereas genotypes HPV-33, HPV-52 and HPV-58 altogether contribute to another 2% of cervical cancer cases [7-9]. The low-risk (LR) HPV genotypes HPV-6 and HPV-11 are responsible for about 90% of genital warts [10].
In Puglia, an Italian region with a population of about four million inhabitants, cervical cancer is the second most common cancer (after breast cancer) in women and about 200 new cases are hospitalized every year (source: Regional Epidemiological Office). Incidence rate of cervical cancer is not available since a regional cancer registry does not exist. Very recently, a cervical cancer-screening program has been set up in the Region for women between 25-64 years of age based on clinical examination and Pap test. The women should be screened at intervals of three years but, at the present, very little data are available on the screening activity, on the frequency of HPV infection and the circulation of the different genotypes within the population.
To date, two types of prophylactic vaccines based on virus-like particles containing L1 capsid protein of HPV have been developed: a bivalent against types 16 and 18 and a quadrivalent one against types 16, 18, 6 and 11 [11, 12].
In the Puglia region, starting from 2009, the bivalent prophylactic vaccine is currently used to vaccinate 12 year-old females (starting from birth cohort of year 1997).

Appropriate and effective preventive strategies need specific epidemiological information and, therefore, it is crucial to know the burden of HPV infection in different geographical areas and the genotypes circulation among local populations also in the view of the evaluation of the impact of vaccination campaigns.
The aim of the present study was to assess the frequency of HPV infection and the genotype distribution of HPV in relation to cytological outcomes, in a sample of women attending the referral outpatient clinics of four Gynecological Departments of the University of Bari (Puglia capital city), prior to the start of HPV vaccination programme.

Methods

Study population

We studied 1,168 women referred for HPV testing to the Molecular Biology Laboratory of the Hygiene Section of the Department of Biomedical Sciences and Human Oncology of the University Hospital of Bari in the years 2005-2008. The women attended the outpatient clinics of four referral Gynecological Departments of the University of Bari either for routine gynecologic care or for Pap test screening. Patients who needed a second level examination following a previous abnormal Pap smear were also enrolled. In all cases HPV-DNA detection was part of the diagnostic algorithm. Only women that gave informed consent to the study and that could provide a copy of written Pap test soon after receiving the result were enrolled in the study.
Cytological evaluation was performed by experienced cytopathologists of reference cytological Services of Departments of Pathological Anatomy of the University of Bari. The cytological Services that perform Pap tests undergo the external quality assurance activities that are part of the regional cancer-screening programme.
The cytological results had been reported according to Bethesda system. The classification was ASCUS for atypical squamous cell of undetermined significance, Low-grade Squamous Intraepithelial Lesion (LSIL), High-grade Squamous Intraepithelial Lesion (HSIL).
Experimental research had been performed with the approval of the ethic committee of the University of Bari and was compliant with Declaration of Helsinki principles.

Sample processing and DNA extraction

Cervical samples, taken by cotton swab or cytobrush, were suspended in a 20 ml preservation solution (Liquiprep); 1-2 ml was pelleted at 600g for 10 minutes. The pellets were frozen at -80°C until the test was carried out. For DNA extraction, the pellets were thawed and resuspended in 200μL of PBS solution (phosphate-buffered saline). The DNA extraction was carried out starting from 200 μL of volume by the use of a commercial kit (High Pure Viral Nucleic Acid and MagNA Pure LC DNA Isolation kit I, Roche Diagnostics, Milan, Italy). The automatic DNA extraction with MagNA Pure was carried out as previously described [13]. The DNA was eluted in a final volume of 100 μL and stored at -20°C.

HPV typing Linear array HPV genotyping

The Linear Array HPV genotyping test (Roche Diagnostics, Milan, Italy) employs the PGMY primers capable of amplifying a DNA fragment of approximately 450 bp in the L1 region. An additional primer pair targets a fragment (268 bp) of the β-globin gene and provides a control for cell adequacy, extraction and amplification. PCR amplification, hybridization reaction and detection were performed according to the manufacturer's instructions. The provided HPV-positive (HPV genotype 16) and -negative controls were used. The Linear Array HPV genotyping system can identify the following HPV types: 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73, 81, 82, 83, 84, IS39, CP6108. The strips were manually interpreted using the manufacturer's reference guide, by reading the individual types down the length of the strip. The kit is CE marked and proved to be highly comparable, useful and accurate for epidemiologic purposes [14].

Statistical analyses

Confidence intervals at 95% (95% CI) were calculated by the modified Wald method. The χ^2-test was used to compare categorical variables. A $p\text{-}value < 0.05$ was considered statistically significant. The χ^2-test for linear trend was used to evaluate differences in the prevalence of HPV infection in different age groups.

Results

A total of 1,168 women were enrolled in the study population. The mean age of the 1,141 women with known age was 37 years (S.D. ± 8.57, range 17-73).
Table I shows the percentage of HPV-positives by age group and cytological results. Overall, HPV-DNA was detected in 30.4% (n = 355, 95% CI: 27.8-33.1). The age-specific prevalence of HPV infection was highest among women ≤ 24 years old (55.8%; 95% CI: 46.2-65.0) and decreased with age, until 45-54 years old (χ^2 for trend = 17.25, $P < 0.0001$). Another peak of 43.7% (95% CI: 28.1-60.7) was observed in women ≥ 55 years old.
The highest rate of HPV infection was found in the age group ≤ 24 both in women with abnormal (64.5%, 95% CI: 52.0-75.3) and with normal cytological results (42.8%, 95% CI: 29.1-57.8) while the lowest rate was found in the age group 45-54 years old (37.7% and 5.2% respectively).
Of the 524 women with an abnormal PAP test (≥ ASCUS), 48.1% (95% CI: 43.8-52.4) were HPV-positive compared to 16.0% (95% CI: 13.4-19.0) of the 644 women with normal cytology.
Table II shows the prevalence of HPV infection in women according to cytological findings. To simplify the analysis, nine women with a diagnosis of carcinoma

were included in the HSIL group. The HPV prevalence increased with the severity of cervical smear abnormality in all age groups.
HPV-DNA was found in 34.4% (95% CI: 25.5-44.5) of women with a cytological diagnosis of ASCUS, in 46.8% (95% CI: 41.8-51.7) of women with LSIL and in 87.0% of women with HSIL/carcinoma (95% CI: 74.0-94.3).
HR-PHR HPV genotypes, alone or in association with other HR-PHR genotypes or LR genotypes, were found in 72.9% (259 out of 355) of infected women.
The 40.6% (144 out of 355) of the HPV positive women had only one HR-PHR genotype and 18.6% (66) had only one LR genotype. Multiple genotypes infections were found in 40.8% (145) of HPV-positive women.
The association of cytological diagnosis with HPV occurrence (HR-PHR or LR) and multiple or single infection in HPV-positives is described in table III. The prevalence of HR-PHR genotypes (alone or in association with other HR-PHR or LR genotypes) increased with the grade of the cytological results. HR-PHR types were found in 78.1% of women with ASCUS, in 78.8% of LSIL and in 87.5% of women with HSIL/carcinoma. In addition, the 55.3% of women with normal Pap test but HPV-positive showed HR-PHR genotypes. Multiple infections were detected in 37.5% of

Tab. I. Frequency of HPV by age group in women with normal and abnormal cytology.

	Cytological finding								
	Normal			Abnormal (≥ ASCUS)			Total (1,168)		
Age groups (years)	No.	HPV+ (%)	95% CI[a]	No.	HPV+ (%)	95% CI[a]	No.	HPV+ (%)	95% CI[a]
≤ 24	42	18 (42.8)	29.1-57.8	62	40 (64.5)	52.0-75.3	104	58 (55.8)	46.2-65.0
25-34	233	40 (17.2)	12.8-22.6	169	84 (49.7)	42.3-57.2	402	124 (30.8)	26.5-35.5
35-44	271	38 (14.0)	10.4-18.7	178	81 (45.5)	38.4-52.9	449	119 (26.5)	22.6-30.7
45-54	77	4 (5.2)	1.6-13.0	77	29 (37.7)	27.6-48.4	154	33 (21.4)	15.6-28.6
≥ 55	5	1 (20.0)	2.0-64.0	27	13 (48.1)	30.7-66.0	32	14 (43.7)	28.1-60.7
Unknown	16	2 (12.5)	2.2-37.3	11	5 (45.4)	21.2-72.0	27	7 (25.9)	11.9-45.0
Total	644	103 (16.0)	13.4-19.0	524	252 (48.1)	43.8-52.4	1168	355 (30.4)	27.8-33.1

[a] 95% Confidence intervals
Chi-square test for linear trend (normal cytology) = 19.69, ***P*** < 0.0001
Chi-square test for linear trend (≥ ASCUS) = 11.80, ***P*** < 0.005
Chi-square test for linear trend (Total) = 17.25, ***P*** < 0.0001

Tab. II. Frequency of HPV infection according to Pap test result and age group.

	ASCUS			LSIL			HSIL[a]		
Age group (years)	No.	HPV+ (%)	95% CI[b]	No.	HPV+ (%)	95% CI[b]	No.	HPV+ (%)	95% CI[b]
≤ 24	4	2 (50.0)	15.0-85.0	53	34 (64.2)	50.7-75.7	5	4 (80.0)	36.0-97.9
25-34	20	6 (30.0)	14.3-52.1	138	68 (49.3)	41.0-57.5	11	10 (90.9)	60.1-100.0
35-44	33	11 (33.3)	19.7-50.4	132	58 (43.9)	35.7-52.4	13	12 (92.3)	64.6-100.0
45-54	24	7 (29.2)	14.7-49.4	45	15 (33.3)	21.3-48.0	8	7 (87.5)	50.8-100.0
≥ 55	11	5 (45.5)	21.2-72.0	7	1 (14.3)	0.5-53.3	9	7 (77.8)	44.3-94.7
Unknown	1	1 (100)	-	10	4 (40)	16.6-68.8	0	0	-
Total	93	32 (34.4)	25.5-44.5	385	180 (46.8)	41.8-51.7	46	40 (87.0)	74.0-94.3

[a] Including 9 women with diagnosis of carcinoma
[b] 95% Confidence intervals

Tab. III. HPV types and prevalence of multiple infections in HPV-positive women.

	Squamous Intraepithelial lesions								Total (N=355)	
	YES (N = 252)						NO (N = 103)			
	ASCUS (N=32)		LSIL (N=180)		HSIL (N=40[a])					
HPV	No.	%	No.	%	No.	%	No.	%	No.	%
HR-PHR	25	78.1	142	78.8	35	87.5	57	55.3	259	72.9
LR	7	21.8	38	21.1	5	12.5	28	27.1	78	21.9
Multiple infections	12	37.5	86	47,7	12	30.0	35	33.9	145	40.8

[a] Including 9 women with diagnosis of carcinoma

women with a diagnosis of ASCUS, in 47.7% of women with a diagnosis of LSIL and in 30.0% of women with a diagnosis of HSIL/carcinoma. The rate of LR genotypes ranged from 12.5% of HSIL/carcinoma to 21.8%of ASCUS.

The frequency of any single HPV genotype among HPV-positive women by cytological diagnosis is shown in Table IV. Overall, 33 different HPV types were identified. Among HR-PHR, the most common HPV genotype was type 16 found in 27.3% of positives followed by type 53 (11.5%), type 66 (9.3%) and type 31 (9.0%). HPV genotype 18 was found in 6.4%. Types 16 or 18 were detected in 33.7% (120/355) of infected women.

HPV type16 was detected in 21.3% of infected women with normal Pap test. The rate of HPV-16 infection increased from 27.7% of LSIL to 45.0% of HSIL/carcinoma. The genotype 53, the second most frequent HR-PHR genotype, was found in 12.6% of women

Tab. IV. HPV type frequency in HPV-positive women by cytological diagnoses.

	Normal benign (103)		ASCUS (32)		LSIL (180)		HSIL (40)[a]		Total (355)	
	No.	%	No.	%	No.	%	No.	%	No.	%
HR/PHR-HPV[b]										
16	22	21.3	7	21.8	50	27.7	18	45.0	97	27.3
18	4	3.8	3	9.3	10	5.5	6	15.0	23	6.4
26	-	-	-	-	-	-	-	-	-	-
31	8	7.7	7	21.7	17	9.4	-	-	32	9.0
33	1	0.9	2	6.2	6	3.3	1	2.5	10	2.8
35	1	0.9	3	9.3	1	0.5	-	-	5	1.4
39	2	1.9	3	9.3	8	4.4	-	-	13	3.6
45	2	1.9	2	6.2	4	2.2	3	7.5	11	3.0
51	9	8.7	1	3.1	17	9.4	3	7.5	30	8.4
52	7	6.7	-	-	9	5.0	1	2.5	17	4.7
53	13	12.6	4	12.5	21	11.6	3	7.5	41	11.5
56	5	4.8	-	-	11	6.1	1	2.5	17	4.7
58	4	3.8	3	9.3	8	4.4	-	-	15	4.2
59	6	5.8	1	3.1	13	7.2	-	-	20	5.6
66	5	4.8	-	-	22	12.2	6	15.0	33	9.2
68	1	0.9	-	-	5	2.7	1	2.5	7	1.9
73	4	3.8	1	3.1	8	4.4	1	2.5	14	3.9
82	1	0.9	-	-	1	0.5	-	-	2	0.5
LR-HPV[c]										
6	10	9.7	-	-	8	4.4	1	2.5	19	5.3
11	4	3.8	-	-	3	1.6	-	-	7	1.9
40	-	-	-	-	-	-	-	-	-	-
42	9	8.7	-	-	17	9.4	3	7.5	29	8.1
54	3	2.9	-	-	6	3.3	-	-	9	2.5
55	4	3.8	-	-	5	2.7	-	-	9	2.5
61	10	9.7	3	9.3	23	12.7	2	5.0	38	10.7
62	11	10.6	3	9.3	16	8.8	-	-	30	8.4
64	-	-	-	-	-	-	-	-	-	-
67	1	0.9	1	3.1	4	2.2	-	-	6	1.6
69	-	-	-	-	-	-	-	-	-	-
70	3	2.9	2	6.2	6	3.3	1	2.5	12	3.3
71	1	0.9	-	-	-	-	-	-	1	0.2
72	1	0.9	-	-	2	1.1	1	2.5	4	1.1
81	4	3.8	-	-	8	4.4	-	-	12	3.3
83	1	0.9	-	-	2	1.1	1	2.5	4	1.1
84	5	4.8	3	9.3	12	6.6	1	2.5	21	5.9
CP6108	9	8.7	4	12.5	14	7.7	1	2.5	28	7.8

[a] Including 9 women with diagnosis of carcinoma

[b] High-risk/Probable high-risk HPV types

[c] Low-risk HPV types

with a normal Pap test and with a significant prevalence also in ASCUS (12.5%), in LSIL (11.6%) and HSIL/carcinoma (7.5%). In women with ASCUS the most frequent HR-PHR genotypes found were type 16 and type 31 (both 21.8%). In women with LSIL, the most frequent HR-PHR genotype, following type 16, was type 66 (12.2%). In HSIL/carcinoma women, other common HR-PHR genotypes, beside type 16, were types 18 and 66 (both 15.0%) followed by types 45, 51 and 53 with 7.5%. Types 16 or 18 were detected in the 33.2% of LSIL and in the 60.0% of HSIL/carcinoma. In particular, among women with carcinoma, 7 out of 9 patients showed type 16, one type 18 and the remaining one type 66.

Among LR genotypes, type 61 was found in 10,7% (38/355) of HPV positive women, type 62 in 8.4% (30/355), type 42 in 8.2% (29/355) and type CP6108 in 7.9% (28/355).

In women with a normal Pap test, the most frequent LR was type 62 (10.6%), in women with ASCUS type CP6108 (12.5%), in women with LSIL type 61 (12.7%) and in women with HSIL/carcinoma type 42 (7.5%).

Discussion and conclusions

This study investigated the frequency and the distribution of HPV genotypes in a sample of women with known cytological results in the Puglia region (South Italy) during the years 2005-2008. The overall prevalence of HPV infection (30.4%) was comparable to that of 31% found in a similar study conducted in Sardinia (Italy) [15]. A previous study conducted in Puglia [16] reported a prevalence of HPV infection in asymptomatic women of 23.1%, though the latter study did not describe the characteristics of the women nor their cytological results and used HPV-DNA detection and genotyping methods (multiplex PCR with four primer cocktails and detection by electrophoresis on 2% agarose gel) completely different from the one we used.

HPV occurrence in women with ASCUS, LSIL and HSIL/carcinoma was in line with the figures reported in other Italian studies [15, 17].

Despite the rate of HR-PHR HPV increases with the grade of the cytological result, the prevalence rates found for these diagnoses were lower than those recently reported in Southern Italy [18], although the percentages in the latter study refer to women with a known histological diagnosis. The low frequency of HPV infection we found in women with a cytological diagnosis of LSIL (47%), despite the use of validated broad spectrum PCR primer sets (PGMY09/11), is concerned. HPV-DNA prevalence values ranging from 55% to 92% have been reported in women with LSIL [19-22]. As previously remarked, a possible explanation could be the clearance of the virus before the resolution of the lesion, the presence of focal lesions not present in the samples tested [23] or, alternatively, false negative results.

Another relevant finding is the absence of HPV-DNA in 6 cases of HSIL/carcinoma and the presence of LR in five case of HSIL/carcinoma. Because nearly all HSIL/carcinoma women should be positive for HPV, the possibility of inadequacy of samples or variability in the cytological assessment may be invoked [22]. It should be also taken into account the possibility that events such as deletions in HPV L1 gene, which is the target of Polymerase chain reaction (PCR)-based test, could affect the detection [4]. The presence of LR in HSIL/carcinoma has been previously reported [18], although further studied to clarify the possible role of HPV type considered at "low risk" in cervical lesions are needed.

The prevalence of HPV infection in women with normal Pap smears (16.0%) was substantially in agreement with previous findings in Italian women with a normal cervix [15, 24].

An elevated proportion of HPV-positive women in our study are infected by HR-PHR genotypes. This emphasizes the need of an appropriate follow-up in women infected by HR-PHR because of the greater probability that the presence of high-risk genotypes can lead to persistent infection and ultimately to cancer of the cervix [5, 25].

A high rate of multiple HPV infections was found in the women studied (about 41% of the HPV positive women). Also other Italian studies showed high prevalence rates, varying from 30.9% to 49.7% [15, 18, 26], but the role of multiple infections in cervical lesions is not clear. However, these data should be considered for a correct evaluation of the impact of the vaccine in our region.

As expected, the most common genotype found was HPV type 16. The second most frequent genotype, among HR-PHR HPV genotypes, was 53. A significant prevalence has also been found in women in the USA [27], where it is the most widespread (2.9% of women) and in Sicily (3.6% of all women) [18]. Also in Spain HPV type 53 seems to be widespread [21].

The distribution of the genotypes according to cytological diagnosis shows that HPV-16 is the most common in every group. For the HSIL/carcinoma group the second most common genotypes were HPV-18 and HPV-66. The genotypes 16, 18 and 66 together, seem to be responsible of 75% of cases of HSIL/carcinoma in the HPV-positive women of the present study. In addition, genotypes 66 and 53 had a significant prevalence also in women with LSIL. Further studies could better clarify the role of these genotypes in our area considering that such a peculiarity has not emerged in other reports in Italy [17, 24, 26, 28] and that vaccines against HPV today in use do not cover these viral types.

In Puglia data on incidence and mortality for cervical cancer are scarce. Screening by Pap test is still on voluntary basis although recently an organized cervical cancer-screening programme has been set up. Also HPV testing is not integrated in the screening programme for cervical cancer in Puglia and it is recommended only in ASCUS triage. Nevertheless, many studies have focused on the importance of HPV testing for cervical cancer screening [29-31]. The high prevalence of HR-PHR genotypes in our study underlines the need for introducing on larger scale HPV testing and also genotyping for

adequate follow up and correct clinical management of HPV infections. In addition, in epidemiologic surveys, type-specific HPV detection could contribute to the evaluation of HPV vaccine effectiveness.

Data on the frequency of HPV infection and on the genotype distribution in Puglia (South Italy) may contribute to assess the impact of HPV vaccination and may provide further information for future decision-making.

Based on our results, potentially, HPV vaccination with the bivalent [16, 18] vaccine in use in Puglia region could prevent about 34% of all infections, 33% of LSIL and 60% of HSIL/carcinoma. It should be considered that the bivalent vaccine has showed a potential of cross-protection effect against types 31 and 45 [32]. Therefore, hypothetical protection provided by vaccination with the bivalent vaccine in our region would reach 46% of all infections, 45% of LSIL and about 67% of HSIL/carcinoma.

The recent introduction of universal HPV vaccination among adolescents will probably modify the epidemiological scenario of HPV infections in the coming years. Further studies should be aimed to evaluate the impact of vaccination in a younger cohort of women and knowledge of HPV type distribution would provide the base for post-vaccination surveillance of circulating viral types and their clinical correlates.

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