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Detection and genotyping of human papillomavirus in gynaecologic outpatients of Messina, eastern Sicily, Italy

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Abstract. In order to determine the prevalence of human papillomavirus (HPV) infection in sexually active female population in Messina, we tested cervical scrapes of women referred to university clinics for routine gynaecologic care. Between March and December 2008, a total of 680 cervical samples of 598 patients (573 Italian from province of Messina and 25 resident aliens) were examined consecutively from laboratory of molecular biology at the Department of Human Pathology. For each sample, cervical cells were collected by centrifugation and DNA was extracted (QIAamp DNA mini kit, Qiagen), followed by a PCR-based HPV DNA assay and reverse dot blot genotyping (HPV-HS Bio plus HPV-strip, AB Analytica or HPV-type, AB Analytica). The overall rate of HPV DNA detection in Italian patients (mean age 34 years; range 15-69) was 70.5% (404/573), with 163 cases of multiple infections (40.3%). In 335 patients (82.9%) a high-risk HPV infection was detected. In this group the coexistence of a low-risk HPV infection was documented in 97 cases while 65 patients exhibited only a low-risk HPV infection. HPV-16 was the most prevalent (33.4%), followed by HPV-6 (28.0%), HPV-31 (24.3%), HPV-58 (11.4%), HPV-66 (11.1%), HPV-53 (6.4%), HPV-18 (6.2%), HPV-56 (5.4%), HPV-33 (5.2%) while the other genotypes identified (HPV-11, -40, -42, -43, -44, -54, -61, -70, -81, -26, -35, -39, -45, -51, -52, -59, -68, -73, -82) were below 5%. HPV prevalence (any type) was 78.7% at age ≤24 years, 73.4% at 25-34 years and 67.1% at 35-44 years and 58.1% at age ≥45 years. A significant association (χ^2=12.718; P=0.006) between HPV DNA detection and the younger age was encountered. Since available data on the prevalence and distribution of HPV infection in Italy are somewhat discordant, this study represents a helpful contribution to the knowledge on the circulation of precise genotypes in east Sicily in order to improve new HPV vaccines.

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Introduction

Human papillomavirus (HPV) constitute a group of more than 120 epitheliotropic DNA viruses that can be identified and classified in different types, based on the sequence analysis of the L1 open reading frame (ORF), which represents the most conserved gene within the genome and encodes the major viral capsid protein L1 (1-4). Fifty HPV types belonging to the genus α-papillomavirus are known to infect the mucosa of the human anogenital tract, determining the onset of benign or malignant epithelial lesions especially in female (2,3,5). These mucosotropic viruses, on the basis of their oncogenic potential, have been classified as high- or low-risk types (6,7). In particular, 18 types have been proposed with a probable (HPV-26, -53, -66) or definite (HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -68, -73, -82) high-oncogenic role (7) and a genital infection with these high-risk HPV is a necessary condition for the development of invasive cervical cancer (8), although other additional factors such as the mutational activation of the K-*ras* gene may be involved in the carcinogenetic process (9,10). On the other hand, infection with low-risk HPV (HPV-6, -11, -40, -42, -43, -44, -54, -61, -70, -72, -81 and CP6108) has been associated with benign condylomatous lesions of the anogenital areas, as well as low-grade squamous intraepithelial lesions of the cervix (3,7). On the basis of these findings, HPV-DNA test has been proposed as a valid aid in the management of women with equivocal cytologic findings before performing additional tests, such as colposcopy (11,12). In addition, HPV testing has been demonstrated to have greater sensitivity for the detection of cervical intraepithelial neoplasia; moreover, the association of an HPV-DNA test to the conventional Pap test reduces the incidence of high-grade squamous intraepithelial lesions of the cervix or cancer, revealed by subsequent screening examinations (13,14).

The prevalence of HPV infections in cervical cytological samples greatly varies in geographical areas and therefore, different genotypes have been encountered in relation to the distribution of patients (7,15-17). However, the precise knowledge on HPV genotype distribution in a given population is

an important finding in order to develop more specific vaccines targeting prevalent HPV genotypes. Since studies on HPV genotype prevalence in Italian female population of oriental Sicily region are not available to date, we thought it would be of interest to report first data regarding HPV-DNA testing from cytological cervical samples of sexually active women referred to University Gynaecological Clinics of Messina.

Patients and methods

Study population. Between March and December 2008, a total of 680 cervical samples of 598 women (573 Italian and 25 alien) were consecutively evaluated for the presence of HPV-DNA in laboratory of molecular biology applied to pathologic anatomy of the Department of Human Pathology of the University of Messina. All samples belonging to Italian women living in province of Messina (eastern Sicily region, southern Italy), with a suspected HPV correlate lesion and referred to university clinics for gynaecologic care, were considered in this study. Overall, 573 woman aged between 15 and 69 years (mean age 34) who underwent for the first time HPV-DNA testing were enrolled and subdivided in four age groups: ≤24 years (mean age 22); 25-34 years (mean age 29); 35-44 years (mean age 39); ≥45 years (mean age 50).

HPV-DNA detection and typing. Cervical specimens were resuspended in 1.5 ml of physiologic solution and stored at -20˚C until DNA analysis or into 20 ml of PreservCyt media (ThinPrep, Cytyc Italia SRL, Rome, Italy) and stored at room temperature. From each sample the cervical cells were collected by centrifugation and DNA was extracted with the QIAamp DNA mini kit (Qiagen GmbH, Germany) and eluted into a final volume of 200 μl, according to the manufacturer's instructions.

Subsequently, 10 μl of nucleic acid were used to detected HPV DNA by nested PCR (HPV-HS Bio or HPV-type, AB Analitica, Padova, Italy) utilizing a Mastercycler gradient (Eppendorf, Hamburg, Germany). For the first-amplification step a combination of degenerate primers were used to amplify a 449-458 bp sequence within the L1 ORF of HPV genome while, for the second step of the nested PCR, biotinylated primers were used to amplify a 139-145 bp sequence. To verify DNA quality, 10 μl of nucleic acid were used to amplify a 202 bp fragment of the Thiosulfate SulfurTransferase (TST) gene using specific primers. For every 10 samples of each PCR, a negative control (H_2O) and a positive control (plasmid clones containing HPV-6, -16, -31, -54 or human DNA) were run to control for possible contamination and accuracy. In order to evaluate the efficiency of amplifications, the PCR products were tested by ethidium bromide staining after electrophoretic migration through polyacrylamide gels.

Finally, HPV typing was performed with a non-radioactive reverse line blot hybridization assay with specific probes for the most frequent HPV-types (HPV-strip or HPV-type, AB Analytica, Padova, Italy). In particular, HPV-strip allows the identification of 8 types (HPV-6, -11, -40/42/69, -43/61/70) considered low-oncogenic risk HPV and 18 types (HPV-16, -18, -26, -31, -33, -35, -39, -45, -51, -52, -53, -56, -58, -59, -66, -68, -73, -82) considered definitive or probable high-oncogenic risk HPV types, while HPV-type, that represent the evolution of HPV-strip kit, allows the identification of 11 types (HPV-6, -11, -40, -42, -43, -44, -54, -61, -70, -72, -81) considered low-oncogenic risk HPV and 18 types (HPV-16, -18, -26, -31, -33, -35, -39, -45, -51, -52, -53, -56, -58, -59, -66, -68, -73, -82) considered definitive or probable high-oncogenic risk HPV types. Hybridisation was performed in a BeeBlot Instrument (Bee Robotics Ltd, Caernarfon, UK) and hybrids between biotinylated PCR products and specific HPV probes were visualized on the strip following the manufacturer's instructions. In some of the cases yielding unidentifiable results by line blot genotyping, the corresponding HPV DNA was sequenced by BMR Genomics (Padova, http://www.bmr-genomics.it).

Statistical analysis. The χ^2 test was used in the statistical analysis. A $P<0.05$ was considered statistically significant.

Results

The overall rate of HPV detection in recruited patients who underwent for the first time to HPV-DNA testing was 70.5% (404/573). The mean age of the HPV positive group was 33 (±9) years, whereas that of the HPV negative was 35 (±11) years. Overall 625 HPV were found in 573 examined samples. Single HPV infection was detected in 241 cases (42.1% of study women and 59.7% of infected ones), while multiple infection with different HPV-types was found in 163 cases (28.4% of study women and 40.3% of infected ones). In particular, the presence of two viruses was encountered in 63.8% of cases with multiple infections, while three, four, five, six or seven viruses were detected simultaneously in 22.7, 7.4, 3.7, 1.8 and 0.6% of cases, respectively.

A high-risk HPV infection was detected in 335 patients (58.5% of study women and 82.9% of infected ones); of these, 238 (71.0%) showed only a single or multiple high-risk HPV infection, while the coexistence with a low-risk HPV infection was documented in 97 cases. A low-risk HPV infection was found in 162 patients (28.3% of study women and 40.1% of infected ones), and 65 (40.1%) of these showed only a single or multiple low-risk HPV infection.

By reverse line blot hybridization assay 27 different HPV-types (18 high-risk HPV and 9 low-risk HPV) were identified in recruited women. HPV-16 was the most prevalent in infected samples (33.4%), followed by HPV-6 (28.0%), HPV-31 (24.3%), HPV-58 (11.4%), HPV-66 (11.1%), HPV-53 (6.4%), HPV-18 (6.2%), HPV-56 (5.4%), HPV-33 (5.2%), while the others genotypes identified (HPV-11, -40, -42, -43, -44, -54, -61, -70, -81, -26, -35, -39, -45, -51, -52, -59, -68, -73, -82) ranged below 5% (Table I). The direct sequencing of 7 infected samples unidentifiable by line blot genotyping permitted to identify other 5 different viruses: the high-risk HPV-67 and the low-risk HPV-cand62 (2 cases), -83, -84 (2 cases), -cand89 (CP6108).

HPV prevalence (any type) was 78.7% (100/127) at age ≤24 years, 73.4% (152/207) at 25-34 years, 67.1% (98/146) at 35-44 years and 58.1% (54/93) at age ≥45 years. A significant association ($\chi^2=12.718$; $P=0.006$) between HPV DNA detection and the younger age was encountered. Single HPV type prevalence per age group is reported in Table I. Distribution of all 625 HPV isolated from 573 samples in their corresponding α species is reported in Table II. In detail,

Table I. Human papillomavirus (HPV) types detected in 573 women in Messina, oriental Sicily, Italy.

HPV types	All women			≤24 years			25-34 years			35-44 years			≥45 years		
	No.	% of study women[a]	% of infected women[a]	No.	% of study women[a]	% of infected women[a]	No.	% of study women[a]	% of infected women[a]	No.	% of study women[a]	% of infected women[a]	No.	%of study women[a]	% of infected women[a]
HR-types	335	58.5	82.9	85	66.9	85.0	131	63.3	86.2	77	52.7	78.6	42	45.2	77.8
16	135	23.6	33.4	32	25.2	32.0	51	24.6	33.6	36	24.7	36.7	16	17.2	29.6
18	25	4.4	6.2	10	7.9	10.0	12	5.8	7.9	1	0.7	1.0	2	2.2	3.7
26	1	0.2	0.2	0	0.0	0.0	1	0.5	0.7	0	0.0	0.0	0	0.0	0.0
31	98	17.1	24.3	21	16.5	21.0	33	15.9	21.7	28	19.2	28.6	16	17.2	29.6
33	21	3.7	5.2	6	4.7	6.0	10	4.8	6.6	3	2.1	3.1	2	2.2	3.7
35	7	1.2	1.7	2	1.6	2.0	1	0.5	0.7	2	1.4	2.0	2	2.2	3.7
39	5	0.9	1.2	1	0.8	1.0	2	1.0	1.3	1	0.7	1.0	1	1.1	1.9
45	6	1.0	1.5	0	0.0	0.0	4	1.9	2.6	1	0.7	1.0	1	1.1	1.9
51	5	0.9	1.2	4	3.1	4.0	1	0.5	0.7	0	0.0	0.0	0	0.0	0.0
52	8	1.4	2.0	3	2.4	3.0	3	1.4	2.0	1	0.7	1.0	1	1.1	1.9
53	26	4.5	6.4	11	8.7	11.0	8	3.9	5.3	3	2.1	3.1	4	4.3	7.4
56	22	3.8	5.4	11	8.7	11.0	6	2.9	3.9	3	2.1	3.1	2	2.2	3.7
58	46	8.0	11.4	13	10.2	13.0	22	10.6	14.5	9	6.2	9.2	2	2.2	3.7
59	8	1.4	2.0	1	0.8	1.0	5	2.4	3.3	1	0.7	1.0	1	1.1	1.9
66	45	7.9	11.1	9	7.1	9.1	22	10.6	14.5	8	5.5	8.2	6	6.5	11.1
67	1	0.2	0.2	1	0.8	1.0	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0
68	2	0.3	0.5	0	0.0	0.0	2	1.0	1.3	0	0.0	0.0	0	0.0	0.0
73	14	2.4	3.5	3	2.4	3.0	7	3.4	4.6	1	0.7	1.0	3	3.2	5.6
82	2	0.3	0.5	1	0.8	1.0	1	0.5	0.7	0	0.0	0.0	0	0.0	0.0
LR-types	162	28.3	40.1	46	36.2	46.0	50	24.2	32.9	43	29.5	43.9	23	24.7	42.6
6	113	19.7	28.0	35	27.6	35.0	33	15.9	21.7	27	18.5	27.6	18	19.4	33.3
11	2	0.3	0.5	2	1.6	2.0	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0
40	1	0.2	0.2	1	0.8	1.0	0	0.0	0.0	0	0.0	0.0	0	0.0	0.0
42	5	0.9	1.2	1	0.8	1.0	1	0.5	0.7	3	2.1	3.1	0	0.0	0.0
44	2	0.3	0.5	0	0.0	0.0	1	0.5	0.7	0	0.0	0.0	1	1.1	1.9
54	4	0.7	1.0	2	1.6	2.0	1	0.5	0.7	0	0.0	0.0	1	1.1	1.9
61	4	0.7	1.0	0	0.0	0.0	2	1.0	1.3	0	0.0	0.0	2	2.2	3.7
cand 62	2	0.3	0.5	0	0.0	0.0	1	0.5	0.7	1	0.7	1.0	0	0.0	0.0
70	4	0.7	1.0	2	1.6	2.0	1	0.5	0.7	0	0.0	0.0	1	1.1	1.9
81	7	1.2	1.7	0	0.0	0.0	3	1.4	2.0	3	2.1	3.1	1	1.1	1.9
83	1	0.2	0.2	0	0.0	0.0	0	0.0	0.0	1	0.7	1.0	0	0.0	0.0
84	2	0.3	0.5	0	0.0	0.0	0	0.0	0.0	2	1.4	2.0	0	0.0	0.0
cand 89	1	0.2	0.2	0	0.0	0.0	0	0.0	0.0	1	0.7	1.0	0	0.0	0.0
40/42/69	15	2.6	3.7	2	1.6	2.0	8	3.9	5.3	5	3.4	5.1	0	0.0	0.0
43/61/70	19	3.3	4.7	8	6.3	8.0	7	3.4	4.6	2	1.4	2.0	2	2.2	3.7
Multiple infection	163	28.4	40.3	45	35.4	45.0	56	27.1	36.8	39	26.7	39.8	23	24.7	42.6

[a]Due to multiple HPV infection, the overall percentage of HPV types exceeds 100%. HR, high-risk; LR, low-risk.

Table II. Distribution in corresponding α species of 625 HPV isolated from 573 cervical samples.

HPV species (types detected)	All women		≤24 years		25-34 years		35-44 years		≥45 years	
	No. HPV isolates	%	No. HPV isolates	%	No. HPV isolates	%	No. HPV isolates	%	No. HPV isolates	%
A1 (HPV-42)	5	0.8	1	0.6	1	0.4	3	2.2	0	0.0
A3 (HPV-61, -cand62, -81, -83, -84, -cand89)	17	2.7	0	0.0	6	2.6	8	5.9	3	3.6
A5 (HPV-26, -51, -82)	8	1.3	5	2.9	3	1.3	0	0.0	0	0.0
A6 (HPV-53, -56, -66)	93	14.9	31	18.0	36	15.4	14	10.3	12	14.5
A7 (HPV-18, -39, -45, -59, -68, -70)	50	8.0	14	8.1	26	11.1	4	2.9	6	7.2
A8 (HPV-40)	1	0.2	1	0.6	0	0.0	0	0.0	0	0.0
A9 (HPV-16, -31, -33, -35, -52, -58, -67)	316	50.6	78	45.3	120	51.3	79	58.1	39	47.0
A10 (HPV-6, -11, -44)	117	18.7	37	21.5	34	14.5	27	19.9	19	22.9
A11 (HPV-73)	14	2.2	3	1.7	7	3.0	1	0.7	3	3.6
A13 (HPV-54)	4	0.6	2	1.2	1	0.4	0	0.0	1	1.2

10 HPV species have been identified and the great majority of HPV types isolated are present in four groups, such as A6, A7, A9 and A10 (Table II).

Discussion

In order to prevent HPV associated cervical lesions as well as cervical cancer, monovalent and polyvalent L1 virus-like particle-based vaccines have been successfully developed (4,18). However, an in-depth knowledge on HPV circulation in specific geographical areas is important to improve strategies connected to HPV vaccination. We report herein, for the first time, data concerning prevalence and genotypes identification of HPV infection in cervical samples from women living in Messina, an eastern province of Sicily, South Italy. HPV-DNA was detected in 70.5% of women aged 15-69 years with a suspected HPV correlate genital lesion, who were referred to university clinics for gynaecologic care. In previous epidemiological Italian studies conducted in representative regions (19) as well as in a selected area of the country (5,20-29), a different overall HPV prevalence has been found, ranging from 6.6 to 61.0%. However, differences in the study population were present among these studies and those based on asymptomatic women recruited into screening program (19,20,23,25) showed a lower incidence of HPV infection than that found in women with abnormal cytology or cervical lesions (5,22,26,28,29). Moreover, the HPV-DNA positivity increased in groups of women with squamous intraepithelial cervical neoplasia (5,29) reaching percentages similar to that encountered by us. The high HPV-DNA positivity documented in the present study might be also due to the diagnostic strategy that has been applied; in fact, we performed a nested PCR based viral detection that represent an highly sensitive method to identify a large number of HPV types infecting the genital tract (5,30) and true HPV prevalence may be underestimated utilizing other HPV detection assays such as single PCR or hybridization on liquid-phase (5,19,31,32). Another finding of our study was the significant (P=0.006) relationship observed between HPV infection and younger patients age. In fact, the prevalence of infection was highest (78.7%) in the group of women aged ≤24 years and showed a continuous decrease with increasing age. This finding is consistent with that reported in other Italian and worldwide epidemiological studies (15,19,20, 23,24,26,27,33) and it is likely related to the transient nature of the infection due to the host immune response as well as to sexual habits.

Among the women living in province of Messina 32 different HPV genotypes were identified by reverse line blot assay or direct sequence analysis, including 19 high-oncogenic

types (HPV-16, -18, -26, -31, -33, -35, -39, -45, -51, -56, -58, -59, -66, -67, -68, -73, -82) and 13 low-oncogenic risk genotypes (HPV-6, -11, -40, -42, -44, -54, -61, -cand62, -70, -81, -83, -84, -cand89). HPV-16, present in 33.4% of infected women, was the most frequently detected genotype and this finding is in agreement with data of other international (4,15) and Italian (5,19-29) studies, confirming thus the worldwide high diffusion of this genotype. The next most common types encountered by us were HPV-6, -31, -58, -66, -53, -18, -56 and -33, with a frequency ranging from 28.0 to 5.2%. In our study, HPV-31 represented the second most common high-oncogenic risk genotype, showing a prevalence of 24.3%, similar to that (25.9%) described in a recent investigation carried out in a Northern Italian population (29). However, this incidence was higher to that encountered in many other Italian studies (5,21-23,26,27) and this might be due to the interference on detection procedures of high intra-type variation described for some genotypes as HPV-31 as well as to the different pattern of geographical distribution of the virus (15,29).

Our data are in keeping with those of a previous study regarding women with abnormal cervical cytology living in Palermo, a Western province of Sicily (26). In fact, in this latter study a total of 42 HPV genotypes were identified and the commonest types other than HPV-16 were HPV-6, -51, -53, -31, -66, -18, -52, -58, -33 and -42, with a frequency in HPV-positive women ranging from 13.4 to 5.5%. In addition to the different number of genotypes identified, probably due to the different diagnostic approach utilized, significant divergences between our and the other Sicilian study (26) were encountered only in the detection rate of four genotypes. In particular, we found a higher prevalence of HPV-6 (28.0 vs. 13.4%) as well as HPV-31 (24.3 vs. 8.5%) and a lower frequency of HPV-51 (1.2 vs. 11.2%) as well as HPV-52 (2.0 vs. 7.6%). The remaining commonest types identified by us showed only small differences in their incidences, restricted in five percentage point, if compared with those found in the other study (26). Therefore, taking into account the complete data, it is evident that only few differences in HPV circulation are present in western and eastern areas of Sicily.

In our study multiple infections with low- and/or high-oncogenic risk genotypes were detected in 40.3% of infected women, most of which showed a double infection. This relevant prevalence reflects the higher sensitivity of nested PCR with reverse line blot hybridization assay in order to detected in the same sample different HPV-DNA, if compared with other diagnostic procedures, such as PCR with sequencing assay. However, a prevalence of one of the genotypes during amplification step cannot be excluded, representing thus an intrinsic PCR limit. Even if it is not clear whether co-infection may favor the development of cervical neoplasia (34,35) a precise detection of all genotypes in an infected cervical sample is important to monitoring the HPV persistence which represent a well known risk factor for cervical cancer.

Of 625 HPVs isolated from our samples 275 (44.0%) were represented by HPV-6, -11, -16 and -18 that are the types against which commercial vaccines have been developed, offering thus type-specific protection against infection. In addition, other 208 HPVs (33.3%) were enclosed into A7, A9 and A10 papillomavirus species, that are the same in the four vaccine types. These HPVs phylogenetically related, sharing between 71 and 89% nucleotide identity within the complete L1 ORF, have presumably similar biochemical and biological properties, including to some extent neutralizing antibody cross-reactivity (2,5). Evidence of partial vaccine cross-protection between highly homologous types such as HPV-16 and -31 as well as HPV-18 and -45 has been documented (36-38), even if this cross-protection is non-existent in other cases (39). Moreover, 22.7% of HPVs isolated from our samples belonged to phylogenetic groups unrelated to vaccine types, showing an absolute antibodies unreactivity. Therefore, this study offers a useful contribution to the knowledge of the HPV circulation in Sicily and it allows to better identify the HPV types prevalent in the oriental region, suggesting the introduction of new targeted vaccines against a wider number of HPV types.

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