

# Prevalence of Multiple High Risk Human Papilloma Virus (HR-HPV) Infections in Cervical Cancer Screening in Lazio Region, Italy

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**Abstract.** *Background/Aim:* It has been well established that human papilloma virus (HPV) is the major cause of cervical pre-cancerous lesions and cervical cancer. Extended HPV genotyping has pointed out that co-infections with multiple high-risk (HR)-HPV genotypes not only is possible and quite frequent, but also has different prognoses. The purpose of this study was to evaluate the prevalence of co-infections in women tested for HR-HPV in the national cervical cancer screening program of Lazio (Italy). *Patients and Methods:* From June 1st to November 30th 2022, we analyzed 30,445 samples of women aged between 30 and 64 years, using the Anyplex TM II HPV HR Detection test by Seegene (Arrow), which identifies 14 HPV genotypes: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68. The data were analyzed using the SG STATS platform. *Results:* In total, 4,244 (13.94%) were positive: 3,290 (77.52%) showed a single genotype infection and 954 (22.48%) an infection with 2 to 5 different genotypes. In 721 (75.60%) cases, two different genotypes were detected, in 191 (20.00%) there were three genotypes, in 41 (4.30%) cases there were four genotypes and in only one case (0.10%) five different genotypes were detected. HPV 16 (262 cases of co-infections) was associated in 27 cases with HPV 31 genotype, in 25 cases with HPV 68 and in 18 cases with HPV 58. *Conclusion:* HPV 16 was the most frequent

genotype detected in co-infections. Immunity status, vaccination, lifestyle, and other possible risk factors, such as the combination of the HR-HPV genotype multiple infections, may influence the development and progression of the disease.

Cervical squamous carcinoma is the fourth most common malignant tumour affecting women worldwide after breast, colorectal, and lung cancer (1). Globally in 2020 the age-standardized incidence of cervical cancer was estimated to be 13.3 cases every 100,000 women/years with a mortality of 7.2 every 100,000 women/years (2).

Although several risk factors can be implicated to the development of cervical lesions, including first sexual intercourse at a young age (<16 years old), multiple sexual partners, smoking, high parity, low socio-economic level, bacterial, co-existence of other sexually transmitted infections, HIV infection or other immunosuppressive states, it is well established that the main cause of precancerous lesions and cervical cancer is a persistent infection with Human Papilloma virus (HPV) (3).

More than 240 HPV types have been identified with a significantly different pathogenicity. Phylogenetic analysis has allowed to distinguish about 37 genera of HPV categorized with a Greek letter and followed by a number that indicates the species (4). HPV of  $\alpha$  genus are virtually the only genus isolated in human cervico-vaginal tissues (4, 5).

High-risk-HPV (HR-HPV), have been identified and studied in screening for prevention of cervical cancer. Although most HR-HPV infections are transient, a small percentage of them persist and progress to high grade squamous intraepithelial lesions (HSIL) and invasive squamous cell carcinoma (SCC) (6). Prospective studies have shown that specific HR-HPV genotypes cause the majority of cervical carcinomas. There are about 14 high-risk HPV genotypes including HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68 according to the National Cancer Institute (5). Two of these, HPV 16 and HPV 18, are responsible for the majority of HPV-related cervical cancers (7, 8).

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Genotypes 16, 31, 33, 35, 52, and 58 belong to genus  $\alpha$  9, genotypes 18, 39, 45, 59 and 68 belong to genus  $\alpha$  7, genotypes 56 and 66 belong to genus  $\alpha$  6, and genotype 51 belongs to genus  $\alpha$  5 (9). It is well established that, among the various genotypes of HR-HPV, the persistent infection is related to the growth of cervical intraepithelial neoplasm (CIN); at least 90% of cervical cancers present DNA sequences of specific HR-HPV genotypes (10). Extended HR-HPV genotyping showed that often multiple genotypes can coexist within the cervical epithelia (11-13). From literature data, it emerges that although some works do not observe a relation between co-infections and cervical lesions (14, 15), several studies have demonstrated that infections with multiple HR-HPV increase the neoplastic transformation risk (16, 17). Furthermore, neoplasms with multiple HR-HPVs could be more resistant to therapy than those with a single infection. This is true mainly in case of co-infections with multiple HR-HPVs belonging to the same genus and with a persistence of the same genotypes (16, 18). Therefore, the epidemiological status of the population, determined by extended HR-HPV genotyping, plays a crucial role in the risk stratification of developing cervical dysplasia (19, 20).

The aim of this study was to evaluate the prevalence of co-infections in women tested for HR-HPV in the national cervical cancer screening program of Lazio, Italy, for better identifying women at a potentially higher risk of developing cervical lesions.

### Patients and Methods

From June 1<sup>st</sup> to 30<sup>th</sup> November 2022, 30,445 samples from females aged 30 to 64 years old were collected according to the program of the Lazio Region, Italy, for the prevention of cervical cancer. Cervical samples were collected using cervix-brush and diluted in ThinPrepR PreservCyt Solution (Hologic, Marlborough, MA, USA).

The samples were processed with 14 HR-HPV genotyping triage using the Anyplex TM II HR-HPV Detection test by Seegene (Seoul, Republic of Korea), Real time PCR method based on DPOTM technology (Dual Priming Oligonucleotides) and TOCETM (Tagging Oligonucleotide Cleavage and Extension), which identifies the 14 HR HPV: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68 (6, 7). The data were analyzed by the SG STATS platform for statistical analysis (Seegene, Seoul, Republic of Korea). Further details are shown in our recently published article (11).

### Results

A total of 26,183 samples were tested negative (86.00%), 4,244 (13.94%) positive, and 18 inadequate (0.06%) (Table I). In total, 3,290 cases (77.52%) showed a single genotype infection and 954 (22.48%) an infection with 2 to 5 different genotypes. Two genotypes were detected in 721 coinfection cases (75.60%), 3 in 191 cases (20.00%), 4 in 41 cases (4.30%), and 5 in only 1 case (0.10%).

Table I. Total samples analyzed.

		%
Samples analyzed	30,427	99.94
Negative cases	26,183	86.00
Positive cases	4,244	13.94
Single infections	3,290	10.81
Co-infections	954	3.13
Invalid samples	18	0.06

Table II. Fourteen high-risk human papilloma virus (HR-HPV) genotypes detected in single infections and co-infections.

Product	Target	Infection type	Number(n)
HR-HPV	16	Single-infection	493
HR-HPV	16	Co-infection	262
HR-HPV	18	Single-infection	133
HR-HPV	18	Co-infection	89
HR-HPV	31	Single-infection	454
HR-HPV	31	Co-infection	250
HR-HPV	33	Single-infection	126
HR-HPV	33	Co-infection	104
HR-HPV	35	Single-infection	96
HR-HPV	35	Co-infection	77
HR-HPV	39	Single-infection	152
HR-HPV	39	Co-infection	141
HR-HPV	45	Single-infection	133
HR-HPV	45	Co-infection	98
HR-HPV	51	Single-infection	225
HR-HPV	51	Co-infection	175
HR-HPV	52	Single-infection	243
HR-HPV	52	Co-infection	170
HR-HPV	56	Single-infection	229
HR-HPV	56	Co-infection	137
HR-HPV	58	Single-infection	233
HR-HPV	58	Co-infection	178
HR-HPV	59	Single-infection	158
HR-HPV	59	Co-infection	102
HR-HPV	66	Single-infection	251
HR-HPV	66	Co-infection	185
HR-HPV	68	Single-infection	364
HR-HPV	68	Co-infection	216

Multiple genotypes were phylogenetically related in 216 cases: 162 cases (75.00%) belonged to the  $\alpha$  9 genus, 45 (20.80%) to the  $\alpha$  7 genus, and nine cases (4.20%) to the  $\alpha$  6 genus. A total of 580 co-infections were phylogenetically unrelated, while in the remaining 158 cases, both genotypes belonging to the same phylogenetic group and from different groups were present. The prevalent genotypes in the multiple infections were: HPV 16 (262; 27.46%), HPV 31 (250; 26.20%), HPV 68 (216; 22.64%), HPV 66 (185; 19.4%), and HPV 51 (175; 18.34%) (Table II). HPV 16 was associated in

27 cases with HPV 31 genotype, in 25 cases with HPV 68, and in 18 cases with HPV 58.

## Discussion

Despite the implementation of cervical cancer screening, invasive cervical cancer continues to be one of the most common cancers among women globally (18). HR-HPV infection is the primary biological risk factor for cervical precancerous lesions and cervical cancer (21), although HR-HPV often fails to integrate into the host cell DNA, giving rise to self-limiting infections (20). If HPV is integrated into the cell genome, it takes approximately 5 years for infection to possibly develop into cervical carcinoma (19). Monitoring and treating the early stages of infection or early lesions may prevent the development of cervical dysplasia and cancer. For these reasons, the Italian government has promoted a free national cervical cancer screening program for women between 25 and 65 years old. Women aged 25-29 years old are screened using primary Pap Test every 3 years, while for women aged 30-65 years old, HR-HPV genotyping is performed every 5 years if the woman shows a negative test (11).

In our region, Lazio, cervical cancer screening is based on extended HR-HPV genotyping. This test is not recommended for women younger than 30 years because there is a higher possibility of acquiring a new HPV infection at a younger age and also a higher chance of self-limiting infections (11, 20).

The extended HR-HPV genotyping was considered a second-level biomarker (22). However, with our method, it is possible to identify multiple viral strains simultaneously in just one step. Our data show that infection with multiple HR-HPV genotypes occurs in 22.48% of positive cases, consistent with other literature data reporting percentages of co-infections ranging from 20% to 45% in infected women (12). Nevertheless, the role of multiple HPV infections in the development of cervical carcinogenesis is still controversial and requires elucidation. Salazar *et al.* suggest that multiple HPV infections may trigger inter-genotypic competition and immune responses, therefore, may not contribute to the development of lesions (15). In contrast, De Brot *et al.* reported an association between multiple HR-HPV infections and persistent low-grade squamous intraepithelial lesion (LSIL), which could help to identify patients at a higher risk of progression to HSIL and SCC (23).

Several studies, however, have shown that HSIL were significantly more frequent in multiple HPV infections than in single ones (24-26). Schmitt *et al.* reported that multiple HPV infections were prevalent in LSIL and HSIL (26). Other studies have shown that although HPV 16 is the major cause of HSIL and SCC, patients with multiple HPV infections also exhibited comparable amounts of different HR-HPV genotypes including HPV 33, 35, 39, 53, 58, 66, and 68 (24, 25). From our data, it can be emerged that HPV 16 is present in the majority of co-infections. It is well established that HPV

16 is the most frequent HPV genotype related to cervical cancer (7, 8). This is probably due to the longer persistence of the infection with HPV 16 compared to other HR-HPV genotypes (12); and it could also be the reason why HPV 16 is the most prevalent genotype in our population (11).

Nogueira Dias Genta *et al.* reported that beyond clinical staging and histological type, multiple HPV genotype infections detected in the same tumor specimen were associated with poorer overall survival (18). Senapati *et al.* showed that risk of cervical carcinoma was associated with multiple genotypes excluding HPV 16 and HPV 18 (16). Trottier *et al.* detected that in addition to HPV 16, co-infections involving HPV 58 may increase cervical oncogenic risk (17).

These conflicting data can probably be explained by studying the phylogeny of the different viral strains. Various studies indicate that infected women with multiple genotypes within phylogenetically related clades have a higher risk of cervical carcinoma compared to those infected with phylogenetically unrelated clades (6, 16). Multiple infections with  $\alpha$  9 genotypes conferred a significantly 5.3-fold higher risk, whereas co-infections with  $\alpha$  7 genotypes conferred a 2.5-fold risk of cervical cancer (16). Same studies show a higher cancerogenic potential of  $\alpha$  9 species than  $\alpha$  7 (12); in agreement with others that show a comparatively higher cancerogenic potential (24). In our study, a clear prevalence (75.00%) of  $\alpha$  9 genus co-infection was observed.

Chaturvedi *et al.* demonstrated that women with multiple infections were at significantly increased risk of both CIN2+ and HSIL+ when compared with women with single infections, with the highest risk in those having multiple oncogenic types of  $\alpha$ -9 species (27). Our results show that in 216 cases, multiple genotypes were phylogenetically related.

Although further studies are needed, the follow up of the persistence of the genotypes and the phylogenetic analysis of multiple infections can be useful in identifying those women who are most likely to develop HSIL and SCC.

## Conflicts of Interest

The Authors report no conflicts of interest in relation to this study.

## Authors' Contributions

Tiziana Pisani: wrote the paper; Maria Cenci: reviewed the paper.

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