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HPV Type Distribution among Romanian Women

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ABSTRACT

Romania has the highest mortality by cervical cancer in Europe, but there are limited data on the spectrum of human papillomavirus (HPV), its etiologic agent, for Romanian women. The main objective of this study was to determine, using modern techniques of molecular virology (Roche HPV *Linear array* genotyping test), the frequency and range of single and multiple HPV infection in a large group of Romanian women [9766 between 15-71 years; from Bucharest (8251 cases, 84.5%) and different regions of the country (1515 cases, 15.5%)]. Most women included in this study, 49.7% (4853/9766 of patients) were detected with different HPV DNA types. Results are heterogeneous, all detectable types being identified. The most frequently found genotypes were HPV 16 (10.2%), HPV 53 (5.9%) and HPV 31 (5.7%). HPV type 18 was the 7th among high-risk types. The most common type of low-risk HPV was 42 (12.6%), types 6 and 11 being on 7th and 13th places. Multiple infection was identified in 25.5% (2359/ 9766) cases, most of them being infected by two different types. HPV infection in the study group has high frequency (49.7%) and specific characteristics. Our herein reported results are part of a larger research study comprising HPV correlations with cytology and biopsies aspects. The study results emphasize the necessity for a cervical precancer screening in Romania and the clinical utility of genotyping. They support the need to develop multivalent prophylactic HPV vaccines.

Keywords: *human papillomavirus (HPV), genotyping human papillomavirus, Linear Array HPV genotyping test, cervical cancer.*

1. INTRODUCTION

Worldwide research has clearly shown that cervical cancer is caused by human papillomavirus (HPV) infection. HPV is a sexually transmitted infection that is very common among young women and men in many parts of the world. Although no effective treatment is available for HPV, the infection is transient in the majority of cases [1-3].A large number of HPV genotypes have been identified, and the mucosal HPV strains are divided into high-risk (HR), medium-risk (MR) and low-risk (LR) categories on the basis of their association with intraepithelial lesions and cervical cancer [4]. Infection by HR HPV types has been demonstrated in almost 100% of cervical carcinoma and it has been shown that persistent infection with the same genotype strongly increases women's risk of developing high-grade preinvasive disease and cancer [5-9]. Numerous international studies have shown that the distribution and prevalence of HPV genotypes is different in relation to the geographical areas. HPV16 is the most prevalent type [2, 10,11]. Cervical cancer is the second most common cancer worldwide, with an estimated 493 000 new cases and 274 000 deaths each year. Cervical cancer is much more common in developing countries, where occurs in 83 % of cases. In developed countries it accounts for only 3.6 % of new cancers. In Europe, the highest mortality rate by cervical cancer is recorded in Romania and Lithuania (13.7 and respectively 10/100 000 women).

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Primary prevention strategies are interventions aimed to avoid or reduce the exposure to the infectious agent and associated risk factors. These are generally based on behavioral, environmental, or biological interventions including immunization programs. Secondary prevention strategies are focused on early detection and treatment of precursor lesions of invasive cervical cancer. Despite the fact that Romania has the highest mortality by cervical cancer in Europe, there are limited data on the spectrum of human papillomaviruses among Romanian women and the necessary precancer screening program is not yet functional.

2. EXPERIMENTAL SECTION

2.1. Patients and specimens. Study design. The retrospective study was conducted in Micomi, a multidisciplinary Clinic specialized in cervical pathology, located in Bucharest. Cervical samples were collected between 2006 and 2011 from 9766 women aged 15 to 71. The analyzed samples were received by the Micomi Molecular Biology Laboratory either from the Micomi Colposcopy Department, or from other gynecology offices in Bucharest and around the country. Most women in the HPV DNA typing study were from Bucharest (8251 cases, 84.5%), the rest resided in different regions of the country: Moldova (685 cases), Muntenia (224 cases), Transylvania (218 cases), Dobrogea (280 cases) and Oltenia (108 cases) (1515 cases, 15.5%).

The description of the HPV spectrum characteristics presented below is part of a larger research study comprising HPV correlations with cytology and biopsies' aspects. The last ones will constitute separated study reports.

The clinical specimens was genotyped using the *LINEAR ARRAY* HPV genotyping test (Roche Molecular Systems), a qualitative *in vitro* test for the detection of 37 anogenital HPV DNA genotypes (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 and CP6108).

2.2. Sample collection. Cervical samples were taken using a Cervex brush which was rinsed in a vial containing PreservCyt solution and transferred to the laboratory for HPV analyses.

2.3. HPV genotyping.

a) DNA extraction. DNA, both HPV and cellular, was released by lysing cervical specimens under denaturing conditions at elevated temperatures in the presence of proteinase K. DNA purification was obtained in columns with a silica-based membrane using vacuum processing [12,13].

b) PCR amplification. The *LINEAR ARRAY* HPV genotyping test uses biotinylated primers to define a sequence of nucleotides within the polymorphic L1 region of the HPV genome that is approximately 450 bp long. A pool of HPV primers is designed to amplify HPV DNA from 37 genotypes. Capture probe sequences are located in polymorphic regions of L1 bound by these primers. An additional primer pair targets the human β globin gene [12,13].

c) Hybridization reaction. Following PCR amplification, the HPV and the β globin amplicon were chemically denatured to form single-stranded DNA by addition of denaturation solution. Aliquots of denatured amplicon were then transferred to the appropriate well of the typing tray that contained hybridization buffer and a single LINEAR ARRAY HPV genotyping strip that was coated with HPV and β globin probe lines. The biotin-labeled amplicon hybridized to the oligonucleotide probes only if the amplicon contained the matching sequence of the complementary probe.

d) Detection reaction. Following the hybridization reaction, the *LINEAR ARRAY* HPV genotyping strip was washed to remove any unbound material. Streptavidin-horseradish peroxidase conjugate was then added to the strip. The streptavidin-horseradish peroxidase conjugate bound to the biotin-labeled amplicon hybridized to the oligonucleotide probes on the strip. The strip was washed to remove any unbound conjugate, and a substrate solution containing hydrogen peroxide and TMB

was added to each strip. In the presence of hydrogen peroxide, the bound streptavidin-horseradish peroxidase catalyzed the oxidation of TMB to form a blue-colored complex, which precipitated at the probe positions where hybridization occurred. The LINEAR ARRAY HPV genotyping strip was then visually read by comparing the pattern of blue lines to the reference guide [12,13].

3. RESULTS SECTION

The proportion of positive and negative results is nearly equal in the study group. From 9766 cases, 4853 cases (49.7%) were HPV positive, and 4913 cases (50.3%) were HPV negative. As expected, we found a large number of positive cases in the under 30 years group (27.9%) and a progressive decrease in the number of positive cases with increasing age (Figure 1). Analyzing the identified types without considering their degree of oncogenic risk, we found that the most common type is high-risk HPV 16 (10.2%), followed by medium-risk HPV53 (5.9%) and high- risk HPV 31 (5.7%). HPV type 58, which, according to the latest meta-analysis of studies published between 1990 and 2010, is on the 3rd place in cervical cancer samples [14], ranks 13 in our study as frequency in overall distribution of types and 5th place among high-risk types (Figure 2). HPV type 18 (3.1%), included with HPV type 16 in two HPV prophylactic vaccines occupies position 15 in the overall frequency.

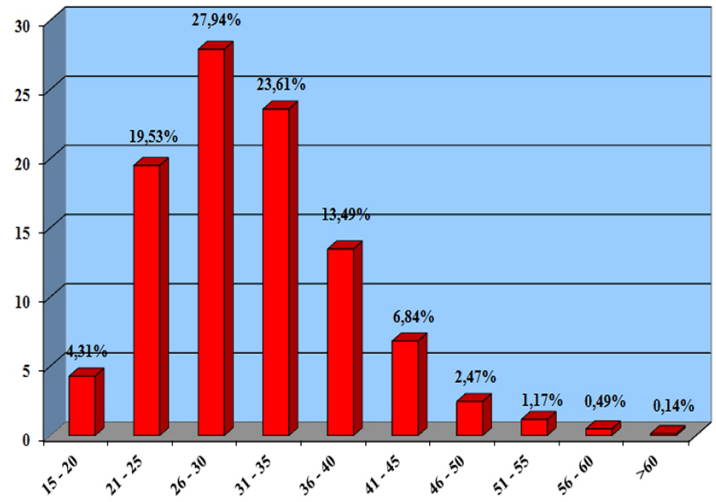

Figure 1: Age distribution of positive HPV patients

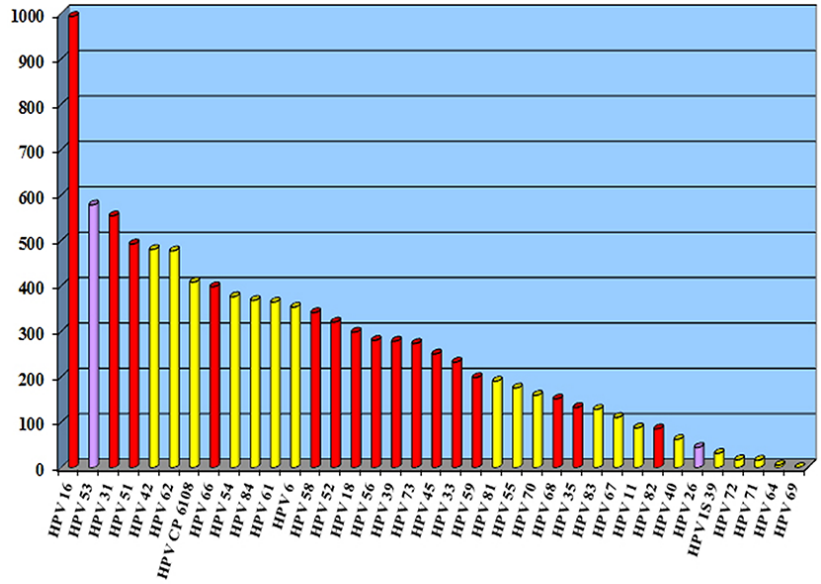

Figure 2: General frequency of high, medium and low-risk oncogenic HPV types

Frequency of high-risk oncogenic HPV types in the study group – the most commonly encountered high-risk oncogenic HPV is 16 (18.8%), followed by HPV 31 (10.5%) and 51 (9.3%). HPV 18, placed 7th place among high-risk types, together with HPV 16 account for about 25% of high-risk HPV infections.

Frequency of low-risk oncogenic HPV types in this study - the most common type of low-risk oncogenic HPV is 42 (12.6%), closely followed by HPV 62 (12.5%) and HPV CP 6108 (10.7%). Types 6 and 11 are on 7th place and 13th place from low-risk oncogenic types, and together have a frequency of about 12%.

Frequency of medium-risk oncogenic HPV types in the study - from oncogenic medium-risk types, HPV 53 is on the first place (table 1).

Table 1: Frequency of high, medium and low-risk oncogenic HPV types

HPV High-risk (HR)	Occurrence	HPV Low-risc (LR)	Occurrence	HPV Medium-risk (MR)	Occurrence
HPV 16	993	**HPV 42**	480	HPV 53	578
HPV 31	554	**HPV 62**	477	HPV 26	31
HPV 51	492	**HPV CP6108**	408		
HPV 66	398	**HPV 54**	376		
HPV 58	341	**HPV 84**	368		
HPV 52	320	**HPV 61**	364		
HPV 18	298	**HPV 6**	353		
HPV 56	280	**HPV 81**	190		
HPV 39	278	**HPV 55**	175		
HPV 73	273	**HPV 70**	159		
HPV 45	250	**HPV 83**	128		
HPV 33	232	**HPV 67**	110		
HPV 59	198	**HPV 11**	87		
HPV 68	151	**HPV 40**	62		
HPV 35	132	**HPV IS39**	44		
HPV 82	85	**HPV 72**	17		
		HPV 71	16		
		HPV 64	5		

Distribution of HPV types by geographic regions. Infection with HPV 16 remains the most commonly detected. The exception is the group of patients from Oltenia region, where the most common high-risk detected type is HPV 66 and HPV 16 ranks in the 8th position. HPV type 53 is the second most common in Oltenia, Transylvania and Bucharest, while in Dobrogea and Moldova we found type 51 on the second place.

Mono-and multiple HPV infection. 24.2% of the HPV-positive cases (2359/9766) were infected with a single HPV type and 25.5% (2494/9766) were infected with multiple types.

Among mono- HPV infection cases, 1363 women had high-risk HPV types, 149 medium-risk HPV types and 847 low-risk HPV types. Distribution of HPV genotypes and their frequency is shown below: HPV 16, HPV 31 and HPV 53 were the most frequent ones (Figure 3).

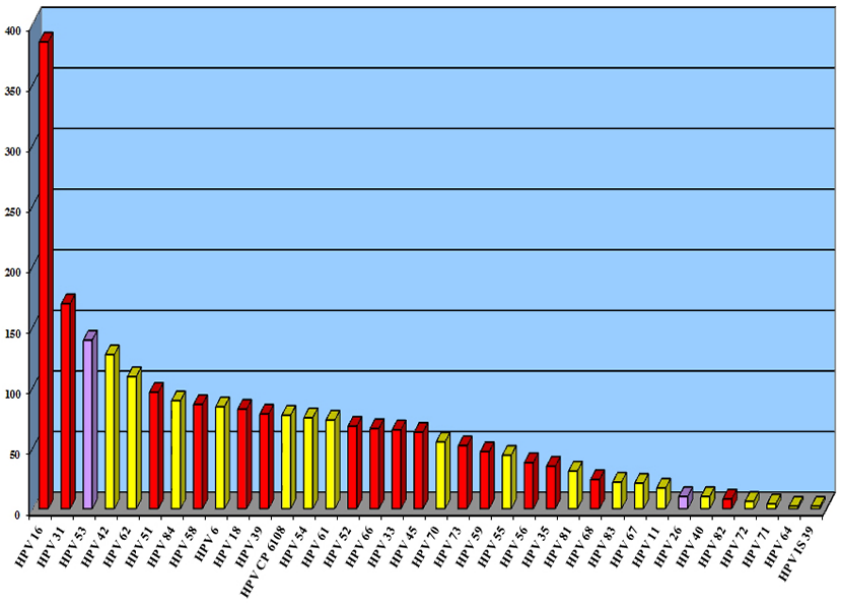

Figure 3: Distribution of HPV types in monoinfection

Multiple infection was identified in about 25% of the HPV-positive cases, patients being infected with 2 up to 12 HPV types. Infection with the combination high-risk and low-risk oncogenic types was the most frequent (1227 patients - 49.2%). 532 (21.3%) women had multiple infection with only high-risk HPV types, 282 (11.3%) with only low-risk HPV types, 148 (5.9%) with the combined high-risk and medium oncogenic types, 109 (4.4%) with medium and low-risk oncogenic types and 196 (7.9%) with all three HPV types. In the study group, infection by two different types were detected in 50.6% of cases, by three types in 25.5%, by four types in 13%, by five types in 6%, by six types in 2.3% and by seven up to twelve types in 2.6% of cases.

Data analysis. Statistical analysis was performed by GraphPad Prism 3.0 calculation program. Each HPV genotype identified was analyzed by considering the number of positive cases and the patients' geographic region. For each type we calculated the mean, median, upper and lower confidence interval (Table 2). To point out the connexion between the data coming from results each genotype and geographic area was used the Spearman correlation test. The p value less than 0.05 was considered to be statistically significant (confidential interval is CI = 95%). In Table 3 one can find the correlation matrices and the corresponding correlation coefficient r^2. We know that a perfect correlation is obtained for $r^2 = 1$ and a total absence of correlation appears whenever r^2 is sufficiently small. In our particular cases we remark a good correlation, the correlation coefficient r^2 being grater than 0.800. The best correlation is between Bucharest with Moldova ($r^2 = 0.947$) and Bucharest with Muntenia ($r^2 = 0.936$). A less correlation (but also statistically significant) is between Dobrogea with Oltenia ($r^2 = 0.708$) and Transilvania with Oltenia ($r^2 = 0.768$). In Table 4 are presented the calculated p values for the corresponding correlated areas. We can remark that all HPV genotypes are strongly correlated ($p < 0.0001$) for the studied areas. The statistic evaluation between the number of co-infecting genotypes and their types (high-risk, HR, medium-risk, MR and low-risk, LR) was realized by using the χ test (Table 5). In the above table we can remark that the number of cases with two genotypes combination (high-risk and low-risk) is significant grater than other cases ($\chi = 696$, $p < 0.0001$). There are some cases for which are frequent two high-risk genotypes and also a three genotypes combination (high and low-risk). The study outcomes rely on the largest number of HPV genotyping done among Romanian women. HPV DNA was found in almost half of the tested cases, which represents a high level of HPV infection, in concordance with the epidemiological data regarding incidence and mortality by cervical cancer. An important

demonstrated parameter of HPV infection is its heterogeneity, all HPV types detectable by the method used being identified. The three main HPV genotypes in the study group were HPV 16 (10.2%), 53 (5.9%), 31 (5.7%). Other prevalent types were HPV 51 (5%), HPV 42 (4.9%), HPV 62 (4%) and HPV CP6108 (4.2%). These findings show differences in comparison with other reports in terms of the percentages of each type. The frequency of HPV 53 and HPV 31 were noticeably higher, while the HPV 18 frequency was lower (3.1%).

Table 2: Statistical analysis of identified HPV genotypes

HPV Genotype	Standard deviation	Average (mean)	Nr.of cases	Upper CI	Lower CI	Median value	Minim value	Maxim value
HPV 16	315.64	165.50	993	185.13	145.87	32.5	4	807
HPV 53	190.74	96.33	578	111.88	80.78	16.0	7	485
HPV 31	185.99	92.33	554	107.82	76.85	15.0	4	471
HPV 42	160.39	80.00	480	94.35	65.65	15.0	5	407
HPV 62	155.55	79.50	477	93.46	65.54	13.5	4	396
HPV 51	153.91	82.00	492	95.60	68.40	18.0	5	395
HPV CP6108	128.41	68.00	408	80.46	55.54	14.0	3	329
HPV 54	123.46	62.67	376	75.15	50.19	10.0	6	314
HPV 66	120.81	66.33	398	78.20	54.46	13.0	9	312
HPV 84	119.47	61.50	369	73.69	49.31	8.5	5	304
HPV 61	117.55	60.67	364	72.74	48.59	11.0	6	300
HPV 58	113.24	56.83	341	68.85	44.81	7.5	1	287
HPV 6	107.32	58.83	353	70.03	47.64	13.5	3	276
HPV 52	97.82	53.33	320	64.05	42.62	12.0	4	252
HPV 18	93.72	49.67	298	60.31	39.03	8.5	3	240
HPV 56	89.24	46.67	280	57.12	36.21	8.0	4	228
HPV 39	88.30	46.33	278	56.71	35.95	8.5	4	226
HPV 73	86.29	45.50	273	55.74	35.26	9.5	2	221
HPV 45	78.22	41.67	250	51.36	31.97	11.0	3	201
HPV 33	70.26	38.67	232	47.71	29.63	8.0	3	181
HPV 59	60.51	33.00	198	41.43	24.57	7.0	4	156
HPV 81	59.21	31.67	190	40.09	23.25	8.5	1	152
HPV 55	57.05	29.17	175	37.62	20.71	5.0	1	145
HPV 70	51.29	26.50	159	34.47	18.53	6.0	2	131
HPV 68	41.78	25.17	151	31.83	18.50	9.5	2	110
HPV 35	42.15	22.00	132	29.19	14.81	2.0	1	107
HPV 83	42.30	21.17	127	28.52	13.81	2.5	0	107
HPV 67	37.73	18.33	110	25.38	11.28	2.0	0	95
HPV 82	27.43	14.17	85	20.00	8.34	3.5	0	70
HPV 11	25.13	14.50	87	19.78	9.22	3.0	1	65
HPV 40	19.49	10.33	62	15.18	5.48	2.5	1	50
HPV IS39	12.97	6.67	40	10.69	2.65	2.0	0	33
HPV 26	9.15	5.17	31	8.39	1.94	0.5	0	23
HPV 72	5.98	2.83	17	5.68	-0.01	0.5	0	15
HPV 71	5.61	2.67	16	5.42	-0.08	0.0	0	14
HPV 64	2.04	0.83	5	2.6	-0.96	0.0	0	5

Table 3: Correlation matrices and the corresponding correlation coefficients

Correlation coefficients	BUCHAREST	TRANSYLVANIA	MOLDOVA
BUCHAREST	-	0.8570634	0.9465925
TRANSYLVANIA	0.8570634	-	0.8130547
MOLDOVA	0.9465925	0.8130547	-
DOBROGEA	0.8635332	0.8575805	0.808692
OLTENIA	0.8452759	0.768153	0.7825236
MUNTENIA	0.9364455	0.8269152	0.889986

Correlation coefficients	DOBROGEA	OLTENIA	MUNTENIA
BUCHAREST	0.863533	0.8452759	0.936446
TRANSYLVANIA	0.857581	0.768153	0.826915
MOLDOVA	0.808692	0.7825236	0.889986
DOBROGEA	-	0.7076439	0.807419
OLTENIA	0.707644	-	0.816391
MUNTENIA	0.807419	0.8163908	-

Table 4 (1): p Values for the correlated areas

p Value	BUCHAREST	TRANSYLVANIA	MOLDOVA
BUCHAREST	-	2.53063E-11	2.76935E-18
TRANSYLVANIA	2.53063E-11	-	1.69445E-09
MOLDOVA	2.76935E-18	1.69445E-09	-
DOBROGEA	1.21309E-11	2.38935E-11	2.41941E-09
OLTENIA	8.84783E-11	4.52682E-08	1.72097E-08
MUNTENIA	4.92522E-17	5.12555E-10	3.84522E-13

Table 4(2): p Values for the correlated areas

p Value	DOBROGEA	OLTENIA	MUNTENIA
BUCHAREST	1.21E-11	8.84783E-11	4.93E-17
TRANSYLVANIA	2.39E-11	4.52682E-08	5.13E-10
MOLDOVA	2.42E-09	1.72097E-08	3.85E-13
DOBROGEA	-	1.39035E-06	2.68E-09
OLTENIA	1.39E-06	-	1.28E-09
MUNTENIA	2.68E-09	1.2824E-09	-

Table 5: The χ test results

Risk/type	Two	%	Three	%	Four	%	Five	%	Six	%
HR	372	7.67	121	2.49	33	0.68	4	0.08	1	0.02
LR	222	4.57	48	0.99	10	0.21	2	0.04	0	0
HR+LR	491	10.12	359	7.4	210	4.33	102	2.1	34	0.7
HR+ MR	98	2.02	31	0.64	13	0.27	4	0.08	1	0.02
MR+LR	78	1.61	20	0.41	9	0.19	2	0.04	0	0
HR+MR+LR	0	0	56	1.15	50	1.03	36	0.74	21	0.43

Risk/type	Seven	%	Eight	%	Nine	%	Ten	%	Eleven	%
HR	1	0.02	0	0	0	0	0	0	0	0
LR	0	0	0	0	0	0	0	0	0	0
HR+LR	14	0.29	10	0.21	5	0.1	1	0.02	1	0.02
HR+ MR	1	0.02	0	0	0	0	0	0	0	0
MR+LR	0	0	0	0	0	0	0	0	0	0
HR+MR+LR	15	0.31	9	0.19	4	0.08	3	0.06	2	0.04

As in other international studies, the first place in our study group is HPV 16. HPV 18, a genotype included in the prophylactic HPV vaccines has the overall position 15 in our study group, and position 7 as occurrence frequency among the high-risk types (5.7%). HPV 53 reported by other studies as having high prevalence in Central and Eastern Europe is placed phylogenetically between high-risk oncogenic types. Still, due to the very low rate of cervical cancer and high frequency in women with normal cytology, warts and low grade intraepithelial lesions, the IARC Working Group and other authors have classified it among medium oncogenic risk types [4,15].

HPV types distribution varies regionally. HPV 16 remains the most common type encountered, except Oltenia region. Type 53 medium risk is the second most common in Oltenia, Transylvania and Bucharest, while in Dobrogea and Moldova second most common is HPV 51.

24.2% of the positive specimens showed only a single genotype on genotyping strips. Considering only "single types", HPV 16, HPV 31 and HPV 53 were the most frequent ones.

Multiple infection (from 2 to 12 different types of HPV) was present in about half of HPV positive cases. The proportion of cases with multiple infection (25.5%) has different values reported by different countries, as 5.3% in Morocco, 6.2% in Southern Italy, 9.8% in Thailand, 14.3% in the Philippines, 16.7% in Paraguay, 28% in Netherlands and 39% in Costa Rica.

The high percentage of multiple HPV infection is clinically significant because of its risk for multifocal lesions of varying severity; each type can cause precancerous lesions or cervical cancer [16, 17].

4. CONCLUSIONS

The present research outcomes show an overall high HPV prevalence among Romanian women tested and some regional specificity in HPV frequency and type, which allow outlining the clinical role of HPV testing and new strategies in cancer interventions field. High virus prevalence, as well as the incidence and mortality by cervical cancer in Romania, emphasize the importance of urgently implementing an organized screening for cervical precancer. In this program, as current international Guide of practice recommends, cotesting women over 30 years by cytology plus high-risk HPV detection should be the best option in evaluating patient's risk for cervical lesions and counterbalancing cytology limitations. When managing individual women with cervical lesions or during post treatment follow up, HPV genotyping test should be preferred, as the effective tool that identifies the type or types of HPV present in patient samples and their implicit risk, as certain cure or monitors a persistent infection.

Due to the demonstrated high frequency of viral infection with genotypes different from those for which current vaccines confer protection, the study results also support the need to develop multivalent prophylactic HPV vaccines.

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