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**Genital Human Papillomavirus infection and genotype prevalence among Albanian women. A cross sectional study.**

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Keywords:	HPV 81, HPV 53, HPV 56, Mediterranean area, Vaccine formulation



Table 1

	General population		Young patients (<30)		P	Mature patients (≥30)	
	N	%	N	%		N	%
<b>Samples</b>	402		99			303	
HPV negative	333	82.84%	70	70.71%		263	86.80%
HPV positive	61	15.17%	25	25.25% **		36	13.20%
Inconclusive <sup>†</sup>	8	1.99%	4	4.04%		4	1.32%
<b>Genotype</b>							
HPV 16	25	40.98%	13	52.0%		12	33.3%
HPV 53	5	8.20%	2	8.0%		3	8.3%
HPV 31	4	6.56%	2	8.0%		2	5.6%
HPV 18	3	4.92%	1	4.0%		2	5.6%
HPV 59	3	4.92%	1	4.0%		1	2.8%
HPV 66	3	4.92%	1	4.0%		2	5.6%
HPV 56	2	3.28%	1	4.0%		2	5.6%
HPV 81	8	13.11%	2	8.0%		6	16.7%
HPV 84	4	6.56%	0	0.0%		4	11.1%
HPV 6	2	3.28%	1	4.0%		1	2.8%
HPV 11	2	3.28%	1	4.0%		1	2.8%

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3 1 **Genital Human Papillomavirus infection and genotype prevalence among Albanian women.**  
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56 31 Running title: genital HR-HPV types circulation in Albania  
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**Abstract**

“High Risk” HPV types have different geographical distribution and evidence suggests their respective prevalence may vary in different areas and regions. An accurate description of High Risk HPV circulation is a key feature for the rational design of prevention and screening campaigns. A cross-sectional, virological study was conducted on adult Albanian women living either in the Tirana area or in the Duresh prefecture. Clinical and gynaecological evaluations were performed according to current standard criteria. HPV detection and typing were carried out by a combined MY09/MY11 and GP5+/GP6+ PCR followed by direct sequencing of generated amplicons. Virological data were obtained from 402 out of 452 patients enrolled between January 2004 and December 2007. Sixty-one patients (15.1 % of the cohort) were found to be infected with a genital HPV. As expected, viral prevalence was higher among women younger than 30 years of age (25.2%) in comparison to those aged 30 or older (13.6%). HPV 16 was found to be the most frequent type (41% of cases), followed by HPV 53 (7.2%), HPV 31 (5.8%) and HPV 18 (4.3%). HPV 81 and HPV 84, were the most prevalent low risk types detected with prevalences of 11.6 and 5.8 %, respectively. No differences were noted in any type specific prevalence between young and mature women. The circulation of HPV types is far more complex than assumed generally. Detailed knowledge of HPV type circulating patterns in specific local geographical areas is essential for appropriate implementation of screening, prevention and surveillance campaigns.

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**Key words:**

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53 HPV 81; HPV 53; HPV 56; Mediterranean area; HPV vaccine formulation; cervical cancer  
54 prevention and screening

For Peer Review

## Introduction

Genital infection with human papillomavirus (HPV) is the most common sexually transmitted disease and represents a major concern for teenagers and young adults. The genital HPVs, presently included in the genus *Alpha-Papillomavirus*, family *Papillomaviridae* (de Villiers et al 2004), can be classified either as High Risk HPV (HR-HPV) or as Low Risk HPV (LR-HPV) based on their association with the onset of cervical dysplasia and cervical cancer (zur Hausen & de Villiers 1994; Walboomers et al. 1999). HR HPVs are represented in human cancers with considerably different prevalences from one type to another. Namely type 16; 18; 45; 31; 33 and 58, account for over 90% of cases whereas the remaining 10% of cases are caused by the many, so called “rare types”(Clifford et al 2003; Clifford et al 2005). Consequently, screening campaigns are under way, diagnostic tools have been devised and vaccine formulations are being proposed to prevent cervical cancer. However, the data available have some serious limitations. Some continents, namely Africa and Asia, are comparatively under-represented while North America, Europe and Australia are conversely over-represented. Within the continents large areas are poorly represented or not represented at all; and finally the vast majority of data are derived from people living in a few major cities within each country and thus, conceivably refer to specific populations and social groups. Nonetheless, there is significant evidence of HR-HPV interregional variation of HPV prevalences. Therefore, based on this context a survey was conducted on HR-HPV types circulating within the Mediterranean area. This is a highly specific region, with a unique climate, crowded with different people with marked different social, economic and cultural conditions and sharing increasingly intense migration exchanges. Thus, conditions promoting and conditions hampering sexually transmitted diseases are simultaneously present and may affect unpredictably HPV circulation. The pattern of genital HPVs circulating in adult females living either in the Tirana area or in the Dures prefecture of Albania is described. In this small country, due to the deep political transformation of the 1990s and to the extensive migratory flux toward Western Europe (Dabalén 2007), a marked increase of cervical carcinoma has been observed (Kule 2005). In addition data on the circulation of HPVs and genotype prevalence are not available. Virological data for preliminary description of local HPV epidemics features are provided. These data are relevant for the implementation of screening and prevention campaigns at a regional level, as well as for a more detailed understanding of HPV infection in Southern Europe and the Mediterranean area.

## Material and methods

**Patients** Patients included in this study were selected from women attending the Gynaecological Out -Patients Clinic of the Oncology Department at the “Nene Terese” University Hospital Centre of Tirana and from women attending the Family Practice at the Gynaecological Clinic of Duress. In order to be eligible, women had to be 18 years of age or older, not pregnant, not to have any history of neoplastic disease, and to have presented solely for routine gynaecological check. The study design and enrolment criteria had been approved previously by the “Nene Terese” University Hospital Centre’s local Ethical Committee.

During the period, January 2004 to December 2007, 452 women were selected among those providing a written informed consent and matching the above inclusion criteria. Enrolled patients were given a standardized questionnaire to obtain data on epidemiological and personal history. This included details about their sexual behaviour, a history of sexually transmitted diseases, tobacco smoking, reproductive history, contraceptive methods used, personal hygiene, and details about their compliance with programmes for cytological screening of cervical lesions.

At entry, enrolled patients underwent a full gynaecological examination, including cytology and colposcopy. Histology was carried out on a selection of patients based on gynaecological indication according to standard criteria.

**Virology** Viral detection and typing were performed on affinity purified total DNA (QIAamp DNA Mini Kit, QIAGEN GmbH, Hilden, Germany) obtained from the residual cytological material. Viral detection was undertaken by the combined use of two different PCR methods. Samples were first assayed using the MY09/MY11 primer pair as described (Manos et al 1989). These highly degenerated primers have been used widely in epidemiological and clinical studies. They generate an approximately 450 bp long amplicon appropriate for precise and sound type identification by downstream direct sequence. As a counterpart this fairly long amplified region makes them rather prone to fail amplification once suboptimal quality DNA is used as the starting material. Considering that part of samples were collected in rural or facilities and that during the transport might have suffered because of cold chain interruption, MY09/MY11 negative samples were retested by the GP5+/GP6+ primers (de Roda Husman et al. 1995). This primer set amplify a roughly 150 bp long fragment and is therefore adequate for analysis of samples with partially degraded DNA. Both reactions were performed in a 50 µl final volume containing 500 nM of each primer, 200 µM of each dNTP (Roche Diagnostics S.p.A – Roche Applied Science, 20052 Monza. italy), 1 unit of thermostable Platinum Taq Polymerase and 1x reaction buffer (both from Invitrogen Life Technologies, s.r.l. - San Giuliano Milanese, Italy). MgCl<sub>2</sub> concentration was 1.5 mM for the MY09/MY11 mixture and 3.5 mM for the GP5+/GP6+ one. Amplification reactions were



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3 119 performed in an IQ4 Thermal Cycler (Bio-Rad Laboratories s.r.l. Segrate, Italy). Amplification  
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5 120 protocol consisted of an initial 150 sec at 95°C for DNA denaturation/polymerase activation step  
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7 121 followed by 35 cycles of annealing for 30 sec at 55°C (for 60 sec at 42°C in the case of GP5+/GP6+  
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9 122 primed amplification), extension for 45 sec at 72°C and denaturation for 30 sec at 95°C, plus a  
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11 123 final cycle with a 10 minute 72°C extension step. Results were evaluated by visual inspection of  
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13 124 Etidium Bromide stained 2,5% agarose gel under UV-B trans-illumination. Adequacy of starting  
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15 125 materials to PCR evaluation was assessed by amplification of a 268-bp fragment of the  $\beta$ -globin  
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17 126 gene as described (Saiki et al 1988). Positive and negative controls consisted in purified DNA  
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19 127 extracted from Siha and from HaCaT cells respectively.  
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23 129 **Viral typing** Viral typing was detected by direct sequencing of amplified products. Briefly,  
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25 130 amplicons were purified by silica gel affinity chromatography (QIAquick PCR purification kit -  
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27 131 QIAGEN), resuspended in a 10 $\mu$ l final volume with 600 nM the upstream primer used for the  
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29 132 amplicon generation (i.e.: either MY11 or GP5+) and transferred to the BIO-FAB RESEARCH  
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31 133 Srl (Pomezia, Italy) where they were sequenced by the BigDye Terminator 1.1 Cycle Sequencing  
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33 134 Kit (Sanger method). Cycle sequencing products have been purified by Sephadex G-50 (GE  
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35 135 Healthcare Bio-Sciences AB) gel filtration. Sequences were analyzed by capillary electrophoresis in  
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37 136 3730 DNA Analyser (Applied Biosystems Inc. Foster City, CA) and aligned to prototype viral  
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39 137 sequences through the BLAST resource at the NCBI (<http://www.ncbi.nlm.nih.gov>).  
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41 138  
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43 139 **Statistical Analysis** Data analysis and graphical work were conducted with the help of Graphpad  
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45 140 Prism 3.0 software pack, (GraphPad Software, Inc., San Diego, CA). The two sided P value was  
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47 141 calculated according to the Fisher's exact test and is reported in table 1 for significant results only.  
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## Results

Patients enrolled ranged in age from 19 to 75 years (median = 36; mean = 38). With regard to education level, 224 patients (49.5%) had a university degree or an equivalent qualification, 173 patients (38.5%) had a high school qualification, 42 (9.2%) had an intermediate school education and 13 (a 2.6%) had no qualification at all or did not give any answer.

**HPV prevalence** The results obtained by the beta-globin amplification indicated that PCR quality DNA was obtained from 402 out of 452 cytological specimens, while no amplification was repeatedly observed in 52 samples (data not shown). These 52 samples were thus filed as inadequate for molecular assay and excluded from further analysis. Among the 402 patients with adequate DNA specimen 61 (approximately 15.1 % of the cohort) were found to be infected with a genital HPV. This figure was higher (25.2%) among women aged between 19-29 and lower (11.9 %) among those aged 30 or over (table I). Young women had a relative risk of genital infection of 2.125, with a Confidence Interval ranging from 1.346 to 3.357.

Table I shows HPV 16, the prototype of the Species 9 of *Alpha-Papillomaviruses*, as the most frequent type accounting for 25 out of 61 typed cases (40.9 %). HPV 53, belonging to species 6 was the second most common HR HPV type being detected in 5 out 61 typed cases (8.2%). HPV 31, the only other member of species 9 found in this cohort, ranked third with 4 out 61 cases (6.5 %). HPV 18 and HPV 59, both members of species 7, were detected in 3 cases each (4.9%), and were followed by HPV 66 (3 cases, 4.9%) and HPV 56 (2 cases, 3.2%) both members of species 6. The two LR types HPV 81 and HPV 84, belonging to species 3, were detected in 8 and 4 cases respectively, with a prevalence of 13.1 and 6.5 %, while the two LR members of species 10, namely HPV 6 and HPV 11, were both detected only in two cases (3.2%). In eight cases post PCR analysis did not confirm the presence of HPV specific sequences in amplified products. These samples were therefore regarded as inconsistent / inconclusive results. Type specific prevalences were also calculated for both young and mature women (as defined above) and are shown in table I.

## Discussion

HPV genital infection and allied dysplastic/neoplastic disease is a major concern for public health representing the most common sexually transmitted disease in western countries and the cause of the second most common neoplasia among women worldwide. However, available epidemiological data are still unsatisfactory with large areas being under-represented or totally neglected (Clifford et al. 2003; Clifford et al. 2005; de Sanjose et al 2007). Indeed, for a number of countries in the mediterranean area only limited and preliminary data are available (Hassen et al. 2003; Lalaoui et al. 2003; Hammouda et al. 2005; De Marco et al 2006), for other countries no data are available at all. This study therefore, provides data on the prevalence of genital *Alphapapilloma Viruses* among a cohort of 402 women living in Albania.

The crude overall prevalence of HPV infection here reported for the whole sample (15.1%), as well as those found among young and mature patients (25.2% and 13.6 % respectively) is within the expected range for unselected patient (Herrero et al. 2000) (Castellsagué 2008) and close to those reported about other Balkan regions (Stojanovic et al 2002) (Mincheva et al 1991), although rather higher than those reported for unselected women in other Southern European countries (de Sanjosé 2007). Conversely, the prevalence of specific HPV genotypes appears very different from the one expected on the basis of global viral circulation data (Clifford et al 2003; Clifford et al. 2005; de Sanjose' et al 2007). Indeed, although HPV 16 confirmed to be the most prevalent type, its prevalence of 40.9 %, while well in tune with the one reported for Greece in a recent large study (Agorastos et al 2009) and similar to the one described in Northern Africa (Clifford et al. 2005; De Marco et al. 2006), is unusually high for unselected patients at global level, as well as in other Southern European countries. The second most prevalent type was HPV 53, which was detected in 5 cases (i.e.: a 1.2% prevalence among the general population and 8.2% among cases). A similar finding is also reported in two recent studies about Greece (Agorastos et al 2009) (Yapijakis et al 2008) where HPV 53 was found the second most prevalent type (14.0 and 12.6 % respectively). HPV53 is a member of alpha-papillomavirus species 6 the oncogenic potential of which is still matter of debate. Indeed, it is considered a HR-HPV on the basis of sequence analysis, while the limited epidemiological data available do not allow the assignment of a definite oncogenic potential. Accordingly, the definition of “probable high-risk” type was suggested (Herrero et al 2000; Munoz et al 2003). Interestingly, two other members of species 6, namely the HR HPV 56 and the “probable” HR HPV 66, were also detected in 3 and 2 cases, respectively. Thus, considering the whole data set, members of species 6 were detected with the notably high frequency of 2.5% in the general population and 16.4% among the 61 infected women. Conversely, the HR types of species 7 (HPV 18; 39; 45; 59 & 70), consistently reported in large studies as the second most

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3 203 relevant contributors to the viral and cervical cancer burden worldwide (Munoz et al 2003; Clifford  
4 204 et al 2003; Clifford et al 2005; de Sanjose et al 2007), are rather scarcely represented in our sample  
5 205 with only HPV 18 and HPV 59 being detected in 3 cases each (with a prevalence of 0.7% among  
6 206 samples and of 4.9% among the cases).

10 207 With respect to LR HPV types, HPV 6, a member of species 10 reported to be frequently  
11 208 present both in low and high grade lesions as well as in cytologically normal specimens, was found  
12 209 with the same prevalences of the less common HPV 11, both detected in 2 cases with prevalences of  
13 210 0.4% and 3.2 % of samples and cases, respectively. Interestingly, eight cases of HPV 81 and four  
14 211 cases of HPV 84 were diagnosed. These two LR types, both members of species 3, with their global  
15 212 19.7% prevalence of cases, represent the third most common species among Albanian women,  
16 213 whereas they are generally very uncommon in other regions, apart from South-Eastern Asia. In this  
17 214 region, close to the Mediterranean basin in terms of climate, genetics and epidemiological features,  
18 215 HPV 81 was reported to be not unusual, ranking fourth among cytologically normal women (de  
19 216 Sanjosé et al 2007). Finally, present data confirm a peak of HPV infection in women aged below  
20 217 30, while no significant differences, in terms of type specific prevalences, were observed in young  
21 218 and mature sub-cohorts in comparison with the whole set of patients, with only minimal changes in  
22 219 the rank of minor types.

33 220 These data were obtained from patients living in two major cities of Albania and  
34 221 characterized by a higher level of education. This might have partially biased the results which thus  
35 222 need to be confirmed by larger, more detailed studies. However, this is the first report, based on  
36 223 existing evidence, dealing with HPV type circulation in Albania, a small country, that is  
37 224 increasingly closing the gap with other European countries and expected to join the EC within the  
38 225 next decade. These preliminary data show once again, that HPV type circulation is far more  
39 226 complex than assumed commonly. Indeed, general features (i.e.: HPV16 is the most prevalent type  
40 227 almost in every region) are associated with regional patterns (i.e.: the relative rarity of HPV 18 in  
41 228 Southern Europe) and with specific features such as the unexpectedly high prevalence of type 53  
42 229 (reported also for neighbouring Greece) and of LR type 81 and 84, thus underlining a number of  
43 230 points: i) supposedly HR HPV “rare types” may be rather frequent in small epidemiological pockets  
44 231 and might cause HR type shift in areas where HPV16/HPV18 based vaccination campaigns had  
45 232 been implemented successfully; ii) vaccination campaigns, formulated on the basis of large scale  
46 233 type prevalences, could be grossly inappropriate leading to a failure of prevention programmes or,  
47 234 worse, boost non-vaccine HR HPV types in the population; iii) detailed knowledge of the HPV  
48 235 genotype circulating patterns in specific local areas is needed urgently to design adequate strategies  
49 236 for the surveillance of vaccinated as well as non-vaccinated women.

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For Peer Review

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**Legends**1  
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Table I

Prevalence of genital HPV infection among Albanian women in the general population and among young (aged between 19-29) and mature patients (aged 30 and over).

The prevalence of specific genotypes among cases is also calculated for the three group of patients

The two sided P value was calculated according to the Fisher's exact test and reported for significant results only (\*\*= P value < 0.005).

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