Human Papillomavirus Infection in Cervical Precancerous Lesions

Gligor Dimitrov[a] PhD, Valentina Sotiroska[a] MSc, Goran Dimitrov[c] PhD, Andrijana Sterjovska[d] MD, Sasho Panov [b] PhD

[a] Center for assisted reproduction and IVF fertilization, General Private Hospital ReMedika, Skopje, Republic of Macedonia

[b]Institute for Biology, Faculty for Natural Science and Mathematics, Ss. Cyril and Methodius University, Skopje, Republic of Macedonia

[c] Clinic for Gynecology and Obstetrics, University Clinical Center, "Ss. Cyril and Methodius" University, Skopje, Republic of Macedonia

[d]Faculty for Medical Science, Goce Delcev University, Stip, Republic of Macedonia

Valentina Sotiroska (Corresponding author)

General Private Hospital ReMedika,

16ta Makedonska Brigada 18, 1000 Skopje, Macedonia

Tel. +389 72 443 170 E-mail: vsotiroska@yahoo.com

Abstract

Infection with high-risk HPV genotypes increases the risk for persistent or progressive cervical lesions. The aim of this study was to determine the frequency and correlation of the HPV infection with the cytological or histopathological diagnosis. A total of 6988 samples were analysed and after exclusion of the repeated samples and/or those containing degraded DNA, 4421 patients were included in the study group. PCR-RFLP technique was used as a method for HPV typing. HPV infection was detected in 1819 out of 4421 patients. The frequency of infection was 23% in the patients' group with cytological and 60% in the group with histopathological diagnosis. The HPV frequencies in precursor lesions were as follows: 51% in patients with mild; 75% in patients with moderate; 91% in patients with severe dysplasia and 93% in *carcinoma in situ* lesions. The most prevalent HPV types in descending order were: HPV 16, 31, 53, etc. HPV infection was the most frequent in patients under 19 years old. In 169 out of 1253 samples with determined viral genotype, a multiple infections were found. This data for the prevalence and distribution of the HPV infection in Macedonian females accentuates the need for the establishment of organized screening programs.

Key words: cervical lesions, human papillomavirus, PCR-RFLP, Macedonia

Introduction

Human papillomavirus (HPV) is a small double stranded DNA virus, belonging to the family *Papillomaviridae* (de Villiers, 2004) that infects epithelial stem cell and causes hyperproliferation of these cells (Pfister, 1984). High-risk HPV E6 proteins target tumor suppressor protein p53 for ubiquitin-mediated proteasomal degradation by interacting with and reprogramming the E6-AP ubiquitin ligase (Gonzalez et al., 2001). Malignant transformation induced by HPV involves inhibition of p53 and pRB tumour-suppressor gene protein products in infected cervical cells by the E6 and E7 viral proteins, respectively. The role of HPV in cervical pathogenesis has been well documented in numerous studies. Currently, more than 40 HPV genotypes have been identified to be associated with cervical cancer (Lorincz et al., 1992). Anogenital HPVs are grouped into "low-risk" usually associated with benign warts and low-grade squamous intraepithelial lesions (such as 6 and 11) and "high-risk" (such as 16, 18, 31, 45, etc) related with precancerous cervical lesions and invasive carcinoma. Viral infection with high-risk HPV genotypes confers an increased risk for persistent infection, progressive cervical lesions and cervical carcinoma.

In the last twenty years the rate of cervical cancer incidence in Republic of Macedonia has been increasing continually, reaching 22.32 per 100.000 females in 2004 (Republic Institute for Health Protection, 2005). The addition of HPV testing may further enhance the accuracy of screening programs. This data confirms the lack of organized screening program for early detection of precancerous cervical lesions.

The aim of this study was to determine the occurrence of HPV infection among the examined population of Macedonian women with cervical abnormalities, using highly sensitive and specific PCR-RFLP method.

Materials and methods

Study population. Cervical specimens were taken at the Clinic for Gynaecology and Obstetrics (University Clinic Center, Skopje) from 6988 patients with abnormal Papanicolaou smears detected during the two years study period at the Laboratory for Molecular Biology (Institute for Biology, Faculty of Natural Sciences and Mathematics, "Ss. Cyril and Methodius" University, Skopje). The mean age of the patients was 35.4 $\pm$ 9.12 (range 13-72) years. The samples from patients with previous surgical treatment of the cervix (conisation, laser vaporisation), repeated samples from patients, and/or samples containing degraded DNA were excluded. Statistical analysis was performed on a study group of 4421 samples. Papanicolaou test was performed using ectocervical and endocervical specimens. Cytological results were classified according revised Bethesda classification (Zerat, 2002, Solomon et al, 2002) as: normal cytology, benign lesions, atypical squamous cells (ASC-US and ASC-H), low-grade squamous intraepithelial lesions (LSIL) and high-grade squamous intraepithelial lesions (HSIL). The majority of the patients with LSIL and HSIL were referred to colposcopic examination and biopsy. According to the morphology determined in the biopsy specimens, the cervical lesions were categorized as: benign lesions, HPV infection and/or mild dysplasia, moderate dysplasia, severe dysplasia, carcinoma in situ lesions and invasive squamous carcinoma (WHO classification). According to the patients' diagnosis the study group was divided into subgroups of patients with cytological and of patients with histopathological diagnosis.

Processing of clinical samples. The cervical cells were collected using a cytobrush and suspended in 3 ml of TBS (Tris-buffered solution) containing antifungal and antibacterial agents. Total DNA was extracted by sodium chloride/choroform extraction followed by ethanol precipitation (Miller et al., 1988; Gemmell & Akiyama, 1996). To assess the quality of the isolated DNA a 536 bp fragment of the ß-globin gene was amplified using the primer pair KM29, 5'-GGTTGGCCAATCTACTCCCAGG-3' and RS42,5'- GCTCACTCAGTGTGGCAAAG - 3' (Wai-Kuen et al., 2003).

HPV detection. HPV DNA was amplified using PCR with degenerate consensus primer pair MY09, 5' - CGT CCM ARR GGA WAC TGA TC - 3' and MY11, 5' - GCM CAG GGW CAT AAY AAT GG - 3' (M=A or C; R=G or A; W=A or T; Y=T or C). The oligonucleotide primers were ordered from Sigma-Genosys, Steinheim, Germany. This primer pair is capable of amplifying a 450 bp DNA fragment from the L1 open reading frame (Jacobs et al., 1997). The PCR was performed in 20 µl reaction mix containing 10 mM Tris-HCl, 50 mM KCL, 3 mM $MgCl_2$, 200 µM of each deoxynucleoside triphosphate, 0.75 µM of each primer, 0.0375 U of Taq DNA polymerase (Sigma-Aldrich), and sample DNA (≈50-300 ng per reaction).

PCR was performed by initial denaturation at 94^0C for 2 min, followed by 35 cycles of: 94^0C for 30 sec, 55^0C for 1 min, 72^0C for 1.5 min, and terminal extension at 72^0C for 3 min on a GeneAmp PCR system 2400 thermocycler. PCR products were resolved by agarose gel electrophoresis and stained with ethidium bromide. All PCR reactions were prepared under laboratory conditions that aim to minimize molecular contamination (Roux, 1995). Positive control (*HPV-TM, 63 bp, TaKaRa)* and negative control (all PCR reagents except sample DNA) were included in each tested series.

HPV typing. HPV types were identified by restriction fragment length polymorphism (RFLP) technique using restriction enzymes *Rsa*I, *Hae*III and *Pst*I, that enables identifying more than 40 types of genital HPV types. Digestion was performed for 3 h in the final volume of 20 µl for each enzyme (15 µl of the digestion mix and 5 µl of the amplified DNA).

Statistical analysis. A database for 6988 samples was generated. Statistical analysis was performed using nonparametric analysis in StatSoft's Statistica v. 6.0 (Chi-square test). Differences were considered significant when p values were < 0.05.

Results

HPV type specific prevalence. In this study, a total of 6988 samples were analyzed. After exclusion of the repeated samples or samples containing degraded DNA, a total of 4421 patients were included in the study group. HPV infection was detected in 1819 (41.14%) samples of which 1253 were typed (Figure 1). HPV was detected but not typed in 567 samples because of the weak electrophoretic signal. The most frequent HPV types in descendant order were: HPV 16 (32.1%), 31 (14%), 53 (12.6%), 18 (9.9%), 58 (5%), etc, (Table I).

HPV age specific prevalence. The incidence of HPV infection was: 70% (30/43) in the patients younger than 20 years; 63% (293/463) in the 20-24 years old; 55% (432/779) in the 25-29 years old; 39% (337/870) in the 30-34 years old; 36% (306/851) in the 35-39 years old; 33% (223/680) in the 40-44 years old; 28% (116/417) in the 45-49 years old; 26% (82/318) in patients older than 49 years (Figure 2).

Multiple HPV infection. Multiple infections were found in 169 (13%) out of 1253 samples with determined HPV genotype. Combination of two low-risk HPV type was detected in 4.7% (8/169) samples, combination of low- and high-risk in 26% (44/169) samples and combination with only high-risk types in 69.2 (117/169) samples. Multiple infections with three HPV types were detected in 3 samples. The most frequent combination was 16 with 31 HPV (9.2%). There was not significant statistical correlation between multiple HPV infection and histopathological diagnosis (chi-square=0.51; p=0.4772).

Cervical diagnosis and HPV infection. The distributions of HPV frequence according to cervical diagnosis are shown in Table II. As expected, women with higher cytology and histopathology grades were associated with higher infection prevalence. Statistical analysis identified significant correlation between the HPV infection and histopathological grade of cervical lesions ($p<0.001$).

Discussion

Cervical cancer has a variable geographic distribution with the greatest incidence in the developing countries (Ferlay et al., 2001; Parkin et al., 1999). Implementation of organized screening programs based on cervical cytology in these countries is expected to significantly lower the number of deaths due to this disease (Franco et al., 1999). According to Vrtacnik and collaborators, early detection and treatment of precursor lesions may be crucial in the prevention of cervical cancer (Vrtacnik et al., 2005).

Depending on the population examined and the method used, the frequency of oncogenic HPV types in various cervical lesions vary significantly (Dybikowska et al., 2002). In this study, the HPV prevalence correlates with previously published data (Camara et al., 2003; Chan et al., 2002; Sasagawa et al., 2001; Shimano et al., 1990). HPV 16 was determined as the most frequent type in all examined groups (n=402; 32.1%). Apart from HPV 16, the other most frequent types were HPV 31 (n=176; 14%), HPV 53 (n=158; 12.6%), HPV 18 (n=124; 9.9%).

In this study the highest frequency of HPV infection was detected in the group of patients under the age of 20 (70% of the 43 analyzed samples), while the lowest frequency was observed in patients over the age of 49 (26% from a total of 318). There is reverse correlation between HPV infection and patient's age which is in accordance with the results of some previously published studies (Monsonego et al., 2005). In the present study the highest frequency of HPV infection was observed in the youngest study group followed by a gradual decrease in the successive older age groups. The broad extent of patient's age (range 13 - 75 years) can be explained by the sexual behavior (promiscuity) of the younger population, compared to the more conservative behavior of the older females in Macedonia.

Prevalence of multiple infections varies in different studies (Vrtacnik et al., 2005; Sasagawa et al., 2001). Comparison of the results is difficult due to the variety of methodological approaches used in their acquisition. In majority of cases, PCR-RFLP technique is suitable for detection of multiple infections. However, in certain cases, the presence of more than two HPV types can lead to overlapping of the multiple electrophoretic bands and to difficulties in the results' interpretation. The ability to identify multiple HPV infection seems to be determined by the starting viral DNA concentration in the samples, as well as the amplification efficiency. Nuovo has stressed that in multiple infections only one HPV type is dominant and all the rest are contaminants that are present in lower concentrations (Nuovo, 1991). In addition, using tissue biopsies samples could lead to more efficient amplification and subsequently to more visible bands, due to the higher DNA yield and quality, in comparison with DNA isolated from cytobrush samples.

Conclusions

The high frequency of HPV infection in Macedonia, as well as the high incidence of cervical cancer, points to the fact that a strategy for decreasing the infection and death rate in women with cervical cancer is urgently needed. The lack of information, the sexual freedom in the young population and a changing life style, all contribute to the increased risk for development of precursor cervical lesions. On the other hand, the geographic location of Macedonia as a transitory country, the turbolent political situation and the presence of foreign military formations in the past years, have contributed to the increased possibility of infection with previously absent or exotic HPV types, unusual for this region. The data for prevalence and distribution of the HPV infection in Macedonia is important for implementation of organized screening program and gynecological doctrine based on evidence.

References:

1. Camara, G., Cerqueira, D., Oliveira, A., Silva, E., Carvalho, L., & Martins, C.R. (2003). Prevalence of human papillomavirus types in women with pre-neoplastic and neoplastic cervical lesions in the Federal District of Brazil. *Mem Inst Oswaldo Cruz, Rio de Janeiro,* 98, 879-883.

2. Chan, P., Mak, K.H., Cheung, J., Tang, N.L., Chan, D.P., & Cheng, A.F. (2002). Genotype spectrum of cervical human papillomavirus infection among sexually transmitted disease clinic patients in Hong Kong. *J Med Virol*, 68, 273-277.

3. Dybikowska, A., Licznerski, P., & Podhajska, A. (2002). HPV detection in cervical cancer patients in northern Poland. *Oncol Rep*, 9, 871-874.

4. Ferlay, J., Bray, F., Pisani, P., & Parkin, D.M. (2001). Globocan 2000: Cancer incidence, mortality and prevalence worldwide, version 1.0. *IARC cancer base,* No. 5. Lyon. IARC Press.

5. Franco, E.L., Villa, L.L., Sobrinho, J.P., Prado, J.M., Rousseau, M.C., Desy, M., & Rohan, T.E. (1999). Epidemiology of acquisition and clearance of cervical human papillomavirus infection in women from a high risk area for cervical cancer. *J Infect Dis*, 180, 1415-1423.

6. Gemmell, N.J., & Akiyama, S. (1996). An efficient method for the extraction of DNA from vertebrate tissues. *Trends Genet*, 12, 338-339.

7. Gonzalez, S.L., Stremlau, M., He, X., Basile, J.R., & Munger, K. (2001). Degradation of the retinoblastoma tumor suppressor by the human papillomavirus type 16 E7 oncoprotein is important for functional inactivation and is separable from proteasomal degradation of E7. *J Virol*, 75, 7583-7591.

8. Jacobs, M.V., Snijders, P.J., van der Brule, A.J., Helmerhorst, T.J., Meijer, C.J., & Walboomers, J.M. (1997). A general primer GP5+/GP6(+)-mediated PCR enzyme immunoassay method for rapid detection of 14 high-risk and 6 low-risk human papillomavirus genotypes in cervical scraping. *J Clin Microbiol*, 35, 791-795.

9. Lorincz, A.T., Reid, R., Jenson, A.B., Greenberg, M.D., Lancaster, W., & Kurman, R.J. (1992). Human papillomavirus infection of the cervix: relative risk associations of the 15 common anogenital types. *Obstet Gynecol,* 79, 328-337.

10. Miller, S.A., Dykes, D.D., & Polesky, H.F. (1988). A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res,* 16, 1215.

11. Monsonego, J., Bohbot, J., Pollini, G., Krawec, C., Vincent, C., Merignargues, I., Haroun, F., Sednaoui, P., Monfort, L., Dachez, R., & Syrjanen, K. (2005). Performance of the Roche AMPLICOR Human papillomavirus (HPV) test in prediction of cervical intraepithelial neoplasia (CIN) in woman with abnormal PAP smear. *Gynecol Oncol,* 99, 160-168.

12. Nuovo, G.J., Darfler, M.M., Impraim, C.C., & Bromley, S.E. (1991). Occurence of multiple types of human papillomavirus in genital tract lesions. Analysis by in situ hybridization and the polymerase chain reaction. *Am J Pathol,* 138, 53-58.

13. Parkin, D.M., Pisani, P., & Ferlay, J. (1999). Estimates of the worldwide incidence of 25 major cancers in 1990. *Int J Cancer*, 80, 827-841.

14. Pfister H. (1984). Biology and biochemistry of papillomaviruses. *Rev Physiol Biochem Pharmacol*, 99, 111-181.

15. Republic Institute for Health Protection. (2005). *Cancer registry in Macedonia (2004).* Skopje, Republic of Macedonia p.26.

16. Roux, K.N. (1995). Optimization and troubleshooting in PCR. In: Dieffenbach CW, Dveksler GS, editors. PCR Primer. *A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. p 53-62.

17. Sambrook, J., Fritsch, E.F., & Maniatis, T. (1989). Molecular cloning. *A laboratory manual, 2nd edition.* New York: Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

18. Sasagawa, T., Basha, W., Yamazaki, H., & Inone, M. (2001). High-risk and multiple human papilomavirus infections associated with cervical abnormalities in Japanese women. *Cancer Epidemiol Biomarkers Prev,* 10, 45-52.

19. Shimano, S., Fukushima, M., & Sawada, Y. (1990). The presence of HPV DNA in squamous metaplasia, dysplasia and carcinoma in situ of the uterine cervix. *Nippon Sanka Fujinka Gakkai Zasshi,* 42, 1331-1338.

20. Solomon, D., Davey, D., Kurman, R., Moriarty, A., O'Connor, D., Prey, M., Raab, S., Sherman, M., Wilbur, D., Wright, T., & Young, N. (2002). The 2001 Bethesda System. Terminology for reporting results of cervical cytology. *JAMA*, 287, 2114-2119.

21. Vrtacnik, Bokal, E., Rakar, S., Mozina, A., & Poljak, M. (2005). Human papillomavirus in relation to mild dyskaryosis in conventional cervical cytology. *Eur J Gynaec Oncol*, 60, 7-12.

22. Ng, W.K., Li, ASM., & Cheung, L.K.N. (2003). Significance of atypical repair in liquid-based gynecologic cytology. *Cancer (Cancer Cytopathol)*, 99, 141-149.

23. Zerat, L. (2002). La nouvelle terminologie de Bethesda: quels changements? *La Revue du Praticien Gynécologie et Obstétrique*. Numéro Spécial 3-10.

Table 1. Prevalence of human papillomavirus types in 1253 females with cervical precancerous lesions (in the period 2003-2005), distributed according to patients age.

HPV type	≤19	20-24	25-29	30-34	35-39	40-44	45-49	≥50	*Total:* *n*	*%*
HPV 6		4	8	3	6	3	1	1	**26**	**2.1**
HPV 11		8	3	9	6		1	2	**29**	**2.3**
HPV 16	6	62	95	88	59	52	23	17	**402**	**32.1**
HPV 18	3	18	29	20	26	23	2	3	**124**	**9.9**
HPV 26		1		1		1		3	**6**	**0.5**
HPV 31	2	28	49	28	28	18	17	6	**176**	**14.0**
HPV 33	1	6	14	5	9	4	2	1	**42**	**3.4**
HPV 35		7	3	1	1	3	1	2	**18**	**1.4**
HPV 39	1	2	3	2					**8**	**0.6**
HPV 42			2		1			1	**4**	**0.3**
HPV 43			1			1			**2**	**0.2**
HPV 44			1		1				**2**	**0.2**
HPV 45		7	13	3	12	4	2	1	**42**	**3.4**
HPV 51		1	3	1		1			**6**	**0.5**
HPV 52		1	2	1		1		1	**6**	**0.5**
HPV 53	5	29	25	33	25	18	14	9	**158**	**12.6**
HPV 54			1	1	1	1			**4**	**0.3**
HPV 55	1	1	2	2	2				**8**	**0.6**
HPV 56	1	5	6	1	2	3			**18**	**1.4**
HPV 58		12	16	13	9	9	2	2	**63**	**5.0**
HPV 59		2	1		1		1		**5**	**0.4**
HPV 61		1	5	3	2		1	1	**13**	**1.0**
HPV 62			2	1			1		**4**	**0.3**
HPV 66		1	7	2			1	1	**12**	**1.0**
HPV 67			1						**1**	**0.1**
HPV 68		4	4	2					**10**	**0.8**
HPV 72				1				1	**2**	**0.2**
HPV 73		5		1	1	1		1	**9**	**0.7**
HPV 81		3	2	1	1	1		1	**9**	**0.7**
HPV 82		1		1		1			**3**	**0.2**
MM4				1		1			**2**	**0.2**
MM7		1		1					**2**	**0.2**
MM8		6	7	2	3	4			**2**	**1.7**
MM9		2	2			1	2		**7**	**0.6**
S039		1		3	1				**5**	**0.4**
CP6108			1	1					**2**	**0.2**
CP8304					1			1	**2**	**0.2**
Total:	**20**	**219**	**308**	**232**	**198**	**151**	**71**	**55**	**1253**	**100**

Table 2. Prevalence of HPV infection according to cervical cytology and histopathological diagnosis in Macedonian women

Diagnosis	HPV negative n (%)	Prevalence of HPV, % HPV positive n (%)	Low-risk HPV n (%)	High-risk HPV n (%)	Multiple HPV n (%)	Uncharacterized HPV n (%)
Cytology						
Normal (27)	21 (77.8)	6 (22.2)	0	2 (100)	0	4 (66.0)
Benign (294)	244 (82.9)	50 (17.1)	6 (22.2)	21 (77.8)	2 (5.4)	23 (46.0)
ASCUS (45)	35 (77.8)	10 (22.2)	1 (20.0)	4 (80.0)	1 (2.7)	5 (50.0)
LSIL (1883)	1450 (77.0)	433 (23.0)	42 (18.9)	180 (81.1)	28 (75.7)	211 (48.7)
HSIL (82)	24 (29.3)	58 (70.7)	3 (10.0)	27 (90.0)	6 (16.2)	28 (48.2)
Total (2331)	1774 (76.1)	557 (23.9)	52 (18.1)	234 (81.9)	37 (12.9)	271 (48.6)
Histopathology						
Benign (330)	183 (55.0)	147 (45.0)	9 (11.7)	68 (88.3)	13 (16.9)	70 (47.6)
HPV infection and/or mild dysplasia (1055)	516 (49.0)	539 (51.0)	48 (12.8)	327 (87.2)	51 (13.6)	164 (30.4)
Moderate dysplasia (404)	10 (25.0)	303 (75.0)	13 (5.0)	245 (95.0)	33 (12.8)	45 (14.8)
Severe dysplasia (205)	19 (9.0)	186 (91.0)	3 (1.7)	169 (98.3)	26 (15.1)	14 (7.5)
In situ carcinoma (84)	6 (7.0)	78 (94.0)	1 (1.3)	74 (98.7)	9 (12.0)	3 (3.8)
Invasive squamous carcinoma (12)	3 (25.0)	9 (75.0)	1 (11.1)	8 (89.9)	0	0
Total (2090)	828 (39.6)	1262(60.4)	75 (7.8)	891 (92.2)	132 (13.6)	296 (23.4)

ASCUS, atypical squamous cells of unknown significance; High-risk HPV (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82, IS039, MM4, MM7, MM9); HSIL, high-grade squamous intraepithelial lesion; Low-risk HPV (6, 11, 40, 42, 43, 44, 54, 55, 61, 62, 70, 71, 72 ,81, CP6108); LSIL, low-grade squamous intraepithelial lesion.

Figure 1. RFLP of the most commonly detected HPV types in Macedonian females with cervical precancerous lesions. Lane 1, 50bp DNA Step Ladder; lanes 2-4: HPV 31; lanes 5-7: HPV 16 and lanes 8-10: HPV 18, all restricted with *Rsa*I, *Hae*III and *Pst*I, respectively

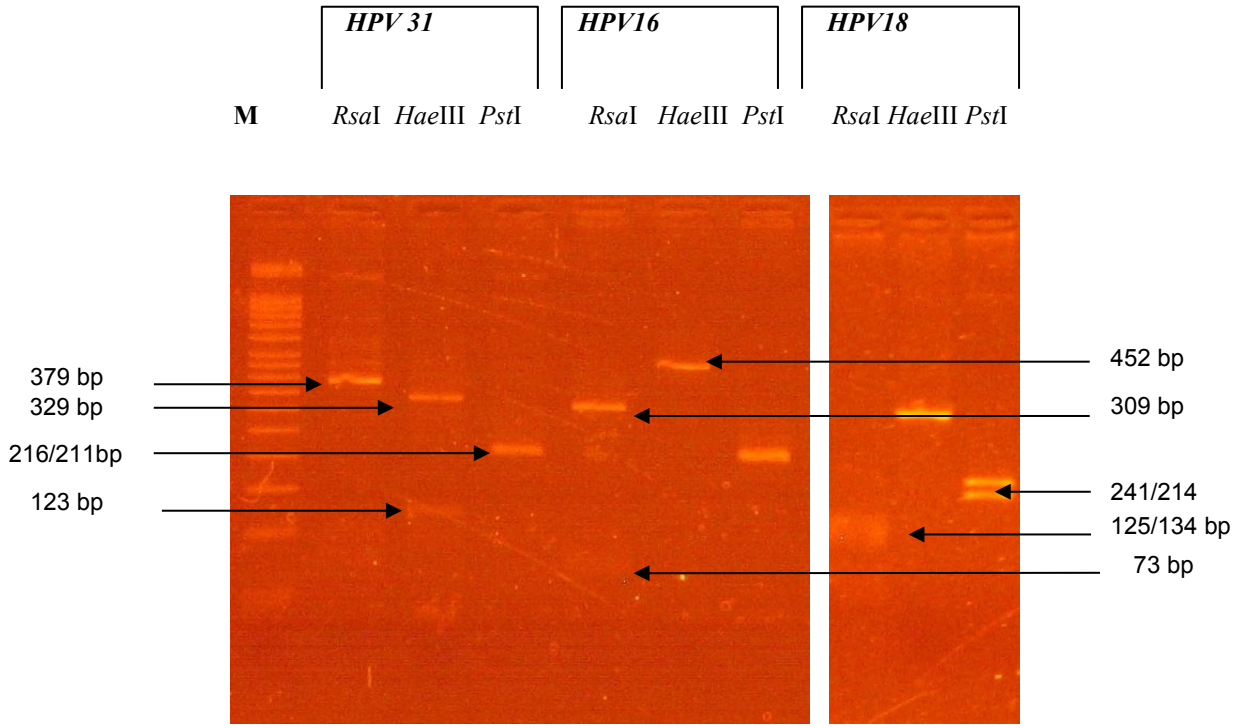

Figure 2. Frequency of HPV infection by age groups in the 4421 Macedonian females with cervical precancerous lesions