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Original scientific paper

Molecular Variants of Human Papilloma Viruses Type 16 and 6 in Women with Different Cytological Results Detected by RFLP Analysis

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A B S T R A C T

HPV infections are common and the presence of the same high-risk type in cervical specimens can be due to reinfection or persistence. Persistent infection is the most important predictor for development of cervical carcinoma. The aim of this study was to validate PCR-RFLP with two sets of primers: MY09/MY11 that amplify a fragment of L1 and P1/P2 that amplify a fragment of E1 ORF. PCR product of MY09/MY11 was digested with a set of 6 restriction enzimes (RE) and PCR product of P1/P2 with a set of 12 RE. Cervical samples from 110 women patients of the University Gynecologic Clinic CHC Zagreb were analyzed. There were 98 (89.1%) PCR positive samples detected with P1/P2 primers, and 94 (85.5%) PCR positive samples detected with MY09/MY11 primers. Seven HPV types were detected with P1/P2-RFLP technique and 17 with MY09/MY11-RFLP. PCR positive samples amplified with both primer pairs agreed with each other in 82 samples; 16 samples were only positive with P1/P2 and 12 samples were only positive by MY09/MY11. HPV 16 was detected in 39 samples with MY09/11-RFLP, out of these two variants (two different patterns) were found with P1/P2 using Dde I, Hae III and Eco I. HPV 6 was detected in 9 samples with MY09/11-RFLP, out of these two variants were found with P1/P2 using HinfI. Combining these two PCR-RFLP methods subtypes of HPV 16 and HPV 6 were detected.

***Key words**: HPV detection, HPV subtyping, RFLP, persistence, reinfection*

Introduction

Human papillomaviruses (HPV) are small DNA viruses that cause proliferation of cutaneous and mucosal epithelia. Over 150 types of HPV are known to date, among these about 40 genotypes infect anogenital region. HPVs can be grouped into low- and high- risk types and some authors also differentiate a third, probably high-risk group[1].

It has been proved that the subsequent development of virtually all cervical cancers requires infection of the uterine cervix with one of high-risk types of HPV[1–4].

However, HPV infections are extremely common in sexually active women and most are transient and benign[4–8].

Unfortunately, some HPV infected women do not spontaneously clear their infections and instead develop persistence. Persistence is defined as the presence of a particular HPV high-risk genotype in two independent cervical specimens obtained 6 month apart. Persistent infection is the most important predictor for the development of cervical carcinoma. The presence of the same high-risk genotype in cervical specimens may be due to reinfection so subtyping (the determination of the HPV subtype) could help in a closer examination of persistence.

Various consensus primer pairs have been used for a polymerase chain reaction (PCR) based method of HPV

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detection. Most commonly used is the MY09/MY11 primer set[9]. Some authors found that the use of multiple sets of PCR primers allow the detection of DNA of HPVs in a higher proportion of cervical neoplasias compared with the use of a single set of PCR primers. It was also shown that PCR for HPV detection using a single set of PCR primers, even the most sensitive one, is not adequate for detecting a broad spectrum of HPV DNA types[10].

The aim of this study was to validate a combined technique of PCR-restriction fragment length polymorphism (RFLP). The first pair of primers used was MY09/MY11. This set of primers is the one validated most often for detecting and genotyping of broad spectrum of genital HPV types. The second pair of primers used was P1/P2. This set of consensus primers is able to promote the amplification of a 526–594 bp fragment of E1 ORF, which contain sequences from different mucosotropic HPV types[11].

Combining these methods a higher sensitivity may be expected, to resolve mixed infections and possibly to find subtypes. The HPV subtypes detecting is very important as a persistence proof.

Materials and Methods

Patients

110 HPV positive cervical samples were taken from women patients of the Gynecologic clinic. Brush swabs were used to obtain the endocervical samples, which were taken during routine gynecologic examination. Samples were taken from women patients of the University Gynecologic clinic CHC Zagreb in the period between 1 September 2003 and 1 October 2005. They were divided in to 2 groups: one group consisted of 40 women with cervical intraepithelial neoplasia (CIN) 1, another of 40 women with CIN 2 or CIN 3. The control group consisted of 30 women with negative cytology. The samples were HPV positive by at least one of the two different general primer PCR-based methods (MY09/MY11 and P1/P2).

Cervical samples were collected in the commercial transport medium HC 2 (Digene Specimen Collection Kit, Silver Spring, Maryland, USA) and were stored at –20 °C until procedure.

All the patients gave informed written consent and the institutional review board approved the study.

Methods

DNA extraction. Total DNA was extracted from commercial transport medium HC 2. Proteinase K (800 μL/mL) and 2 μL of Tween-20 were added to the sterile micro tube with 200 μL of samples. This mixture was placed in a thermo block at 55 °C for at least 2 hours. Proteinase K was afterwards inactivated at 95 °C for 10 minutes. DNA concentration and quality were determined by 1% gel electrophoresis.

HPV detection by PCR. The isolated DNAs were tested for the presence of HPV DNA by using general primers PCR-based method. Degenerated consensus primer pair MY09/MY11 amplifies an approximately 450 bp fragment in the L1 ORF. The other primer pair (P1/P2) amplifies a 526–595 bp fragment in the E1 ORF.

HPV DNAs in specimens were amplified using PCR primer pairs MY09/MY11 and P1/P2 as previously described[9,11]. A 260-bp fragment of the cellular β-globin gene was also amplified as previously described[12]. Successful amplification of the β-globin gene fragment indicated that the DNA sample was adequate for HPV DNA genotyping analysis and that no PCR inhibitors were present. The specimens with negative β-globin amplification were not taken into consideration.

HPV genotyping. To determine HPV type, PCR products of amplification with MY09/MY11 were digested with restriction enzymes and afterwards were analyzed by agarose gel electrophoresis, as previously described[9]. PCR products of amplification with P1/P2 primer pair were digested with 12 restriction endonucleases (RE) (BamH I, Dde I, Hae II, Hinf I, Pst I, Rsa I, Alu I, Nsi I, Hpa II, Bgl II, Ecor I, Kpn I) and were afterwards also analyzed by agarose gel electrophoresis. The PCR product of MY09/MY11 was digested with 6 RE and the PCR product of P1/P2 was digested with 12 RE.

Results

The study comprised cervical samples taken from 110 women which were HPV positive by at least one of two different general primer PCR-based methods: MY09/MY11 and P1/P2. There were 98 positive samples with the set of general primers P1/P2; the sensitivity of the method was 89.1%. There were 94 (85.5%) PCR positive samples with the primer set MY09/MY11. PCR positive samples amplified by both primer pairs agreed with each other in 82 cases. Sixteen (16) samples were only positive by P1/P2 and 12 samples were only positive by MY09/MY11 (Table 1).

TABLE 1
PCR PRIMERS USED AND NUMBER OF WOMEN WITH DIFFERENT CYTOLOGICAL RESULTS

Primer pair		No. of women with different cytological results			
MY09/MY11	P1 / P2	Normal CIN 1	CIN 2 and / or 3		Rates of detection (%)
+	+	17	33	32	82 (74.6%)
–	+	7	4	5	16 (14.5%)
+	–	6	3	3	12 (10.9%)
Total		30	40	40	110 (100 %)

Out of the 30 control samples (women with normal cytology), in 5 samples (16.7%) the quantity of DNA was too low for genotyping by the RFLP method. Therefore, a total of 105 cervical samples were genotyped and 17 different genotypes were found (Table 2).

The most common genotypes were HPV 16 (37.1%), HPV 31 (8.6%) and HPV 6 (8.6 %). Six samples remained

TABLE 2
DISTRIBUTION OF HPV TYPES IN WOMEN WITH DIFFERENT CYTOLOGICAL RESULTS

Type	No of women with different cytological results			Total	N %
	Normal	CIN 1	CIN 2 or / and CIN 3		
6	6	3		9	8.6
11	3			3	2.9
16	5	10 p=1.0000	24 p=0.0198	39	37.1
18		2	2	4	3.8
31	2	3	4	9	8.6
33	1		2	3	2.9
39			1	1	0.9
40	2	3		5	4.8
52			2	2	1.9
53	1	3	1	5	4.8
56		2		2	1.9
58	1	3		4	3.8
61	1			1	0.9
62	1	2		3	2.9
73			1	1	0.9
X	2	3	1	6	5.7
16 + 70		1		1	0.9
33 + X		1	2	3	2.9
54 + X		2		2	1.9
58 + X		2		2	1.9
Total	25	40	40	105	100.0

untyped (HPV X – 5.7 %) and in 8 samples (7.6 %) mixed infections were found (Table 2).

Amplified sequences were analyzed by restriction fragment length polymorphism (RFLP). Seventeen different types were found with MY09/11-RFLP. Seven different patterns (types) were found with RFLP method performed on E1 ORF.

HPV 16 was detected in 39 samples with MY09/11-RFLP (standard method).

TABLE 3
VARIANTS (SUBTYPES) OF HPV 6 AND HPV 16 COMPARING WITH DIFFERENT GROUPS OF WOMEN

Subtypes*	No of women with different cytological results			Total
	Normal	CIN 1	CIN 2 3	
HPV 16-a	2	7	7	16
HPV 16-b	3	3	17	23
HPV 6 -a	2	2	0	4
HPV 6-b	4	1	0	5

*PCR-RFLP after amplification with p1/p2

Out of the 39 samples determined as HPV16 by the standard method, the use of P1/P2 primers gave 2 different patterns after restriction. The HPV16a pattern was found in 16 samples and the HPV16b pattern in 23 samples. The restriction method was carried out with 12 enzymes after P1/P2 amplification, but the following enzymes were sufficient to prove these subtypes: *AluI, HaeIII, EcoRI* and *Dde*. Using restriction with *AluI* gave one fragment in HPV16a, and digested HPV16b into 2 fragments. *HaeIII, EcoRI* and *Dde* digested HPV16a, but there was no restriction in HPV16b.

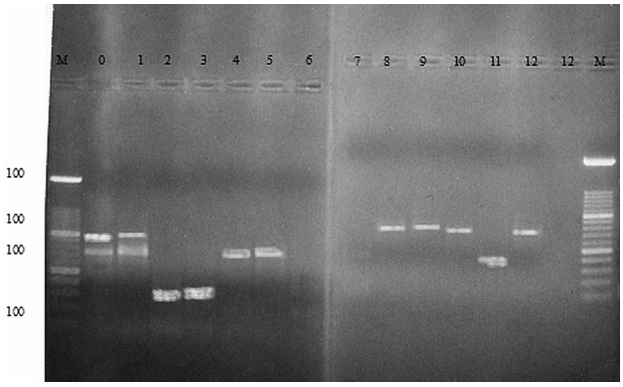

Fig. 1. RFLP patterns of PCR products amplified with primer pair p1/p2 HPV 16 (variant 1). Agarose gel electrophoresis of PCR products digested with different restriction enzymes. Lane M: 100-bp ladder. Lane 1: BamH I (undigested), lane 2: Dde I (digested), lane 3: Hae III (digested), lane 4: Hinf I (undigested), lane 5: Pst I (undigested), lane 6: Rsa I (digested), lane 7: Alu I (digested), lane 8: Nsi I (undigested), lane 9: Bgl I (undigested), lane 10: Hpa II (undigested), lane 11: EcoR I (digested), lane 12: Kpn I (undigested).

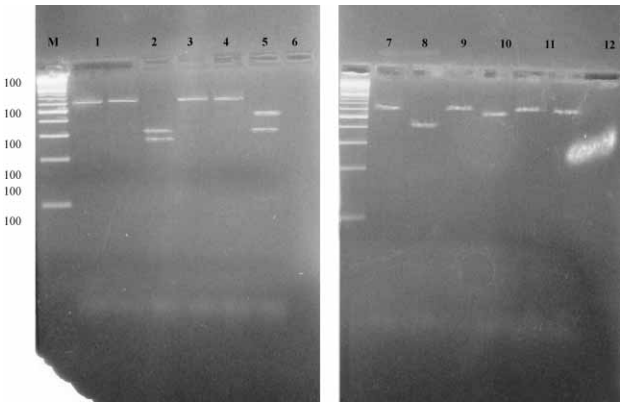

Fig. 2. RFLP patterns of PCR products amplified with primer pair p1/p2 HPV 6 (variant 2). Agarose gel electrophoresis of PCR products digested with different restriction enzymes. Lane M: 100-bp ladder. Lane 1: BamH I (undigested), lane 2: Dde I (undigested), lane 3: Hae III (digested), lane 4: Hinf I (digested), lane 5: Pst I (undigested), lane 6: Rsa I (digested), lane 7: Alu I (undigested), lane 8: Nsi I (digested), lane 9: Bgl I (undigested), lane 10: Hpa II (undigested), lane 11: EcoR I (undigested), lane 12: Kpn I (digested).

HPV 6 was detected in 9 samples with MY09/11-RFLP, and restriction after P1/P2 amplification gave 2 patterns. The HPV6a pattern was found in 4 samples, and HPV6b was found in 5 samples. The *HinfI* and *KpnI* enzymes digested amplification product and this led to the determination of HPV6a, while HPV6b had no digestion. There were no differences in restriction patterns for the other 10 enzymes (Table 3, Figure 1, 2).

Discussion

The PCR-RFLP strategy enables the detection and typing of all known and yet unknown genital HPVs. However, detection of new HPV genotypes, variants and subtypes remains difficult when using type-specific primers or probes. Generic primers for conserved regions, such as the L1 (MY09/11) are very useful for the detection of these viruses[10]. The rates of detection of HPV DNA with only one generic primer pair are not satisfactory[10,13]. This study validated the combination of two sets of consensus primers (MY09/MY11 and P1/P2). MY09/MY11 is one of the most commonly used sets of degenerate primers, which amplifies the conserved region L1 mentioned above[9,14]. The other set of consensus primers was P1/P2. With the last one primer set it is possible to promote the amplification of a 526–594 bp fragment of E1 ORF, which contains sequences from different mucosotropic HPV types[12]. To the best of our best knowledge, no other author has used this combinations of primers.

This combination provided a higher sensitivity in HPV detection than the use of one primer pair alone. This difference was not statistically significant (χ-square test $p=0.1824$), in contrast to the similar research made by Kado and coworkers[10]. In their study they used as many as five different sets of PCR primers and achieved a significantly higher rate of HPV detection. Namely, only 34 of 77 (44%) HPV DNAs were amplified by all of the primer pairs used. The combined overall detection rate was even 76/77 (99%). The combination of five methods is labor intensive and too expensive for routine work.

It was also expected that combining the previously described methods would result in finding more different HPV types than with one method, lead to resolving some mixed infections and probably to finding subtypes (variants) of some HPV types.

Although 12 different restriction enzymes were used in this research, the RFLP method performed on E1 ORF was weakly discriminatory in comparison with the most commonly used RFLP method (MY09/ MY011). The intention of this study was to screen the enzymes on the part of the gene E1 and choose those that digest the region of interest. This method resulted finding in 7 different types (out of 17 that were typed with MY09/11-RFLP). A recently published study described a PCR-RFLP-based method which permits discrimination of all known mucosal HPV types (49 HPV types and 2 subtypes)[15]. This range of HPV identification greatly exceeds the number of types characterized by any currently available commercial assays or by any other genotyping methodology based on RFLP analysis[9,16–18].

In epidemiological studies, sequence variations can be used as a marker to track the spread of the virus in contact networks through populations[19,20]. HPV 16 is known to have a number of variants, each with a different geographic distribution. Some are more often associated with invasive neoplasias. HPV variant classes and subclasses of the HPV 16 were identified by sequencing regions of the E6, L1 and E2 genes[21–26].Variants of some other high-risk types were also found (HPV 18, HPV 31, HPV 35, HPV 68)[21,23].

HPV infections are extremely common and the presence of the same high-risk type in cervical specimens could be due to reinfection. Subtyping (the determination of the HPV variants) could help in a more precise investigation of persistence, as the most important predictor for the development of cervical carcinoma.

Two variants of HPV 16 and HPV 6 were found by combining two RFLP methods. The restriction method was carried out with 12 enzymes after P1/P2 amplification, but the following enzymes were sufficient for proof of the HPV 16 subtypes: AluI, HaeIII, EcoRI and Dde. The HPV 6 subtypes were determined with enzymes HinfI and KpnI. For the other 10 enzymes there were no differences in digestion.

Future studies are also required to provide comprehensive information about the significance and potentional role of these E1 variations of HPV 16 among different groups of women (normal, CIN I, CIN II/III). In this study HPV 16b was found in 17 samples obtained from women with CIN2 and 3, while HPV 16a was found with 7 women with CIN2 and 3. This research was performed on a relatively small number of samples and further research on a larger number of samples is necessary.

In conclusion, a higher degree of HPV detection is achieved by combining these two PCR-RFLP methods than by using one pair of primers alone. This method is much easier to perform than sequencing, so it could be used as an alternative method for HPV subtyping and also for epidemiological research. We did not find any literature describing HPV 16 and HPV 6 subtype detection using the RFLP method. The importance of HPV 16 subtypes is more obvious, for example, in the detection of persistence, while the importance of HPV 6 subtypes is less well known. Grassman and his collaborators did not find a link between HPV 6 variants and different oncogene activity of the promoter[27,28].

Because these viruses have extraordinary genetic variability, it is important to have simple and easy methods for detecting subtypes than can be uncovered by current vaccine. The possibility of infection with several molecular variants of specific HPV type at the same time should also be studied, and whether natural genetic variability occurs during persistent infection. Therefore, further research should focus on the currently available molecular methods for HPV typing and the evaluation of RFLP restriction efficacy in different HPV regions.

REFERENCES

1. MUNOZ N, BOSCH FX, DE SANJOSE S, HERRERO R, CASTELLAGUE X, SHAV KV, SNIJDERSPJ, MEIJER CJ, N Engl J Med, 348 (2003) 518. — 2. ZEHBE I, WILANDER E, J Pathol, 181 (1997) 270. — 3. ZUR HAUSEN H, Biochim Biophys Acta, 1288 (1996) F55. — 4. BRENTJENS MH, YEUNGYUE A, LEE PC, TYRING SK, Dermatol Clin, 20 (2002) 315. — 5. EVANDER M, EDLUND K, GUSTAFSSON A, GUSTAFSSON A, JONSSON M, KARLSSON R, RYLANDER E, WADELL G, J Infect Dis, 171 (1995) 1026. — 6. VAN DOORNUM GJJ, PRINS M, JUFFERMANS LHJ, Genitourin Med, 70 (1994) 240. — 7. CARR J, GYORFI T, Clin Lab Med, 20 (2000) 235. — 8. WIKSTROM A, POPESCU C, FORSLUND O, Int J STD AIDS, 11 (2000) 80. — 9. BERNARD H-U, CHAN S-Y, MANOS MM, ONG KC, VILLA HD, PEYTON CL, BAUER HM, WHEELER CM, J Infect Dis, 170 (1994) 1077. — 10. KADO S, KAWAMATA Y, SHINO Y, KASAI T, KUBOTA K, IWASAKI H, FUKAYAWA I, TAKANO H, NUNOYAMA T, MITSHUHASHI A, SEKIYA S, SHIRASAWA H, Gynecol Oncol, 81 (2001) 47. — 11. CONTORNI M, LEONCINI P, J Virol Methods, 41 (1993) 23. — 12. SAIKI RK, SCHARF S, FALOONA F, MULLIS KB, HORN GT, ERLICH HA, ARNHEIM N, Science, 230 (1985) 1350. — 13. GRCE M, HUSNJAK K, SKERLEV M, LIPOZENČIĆ J, PAVELIĆ K, Anticancer Res, 20 (2000) 2097. — 14. MANOS MM, TING Y, WRIGHT DK, LEWIS AJ, BROKER TR, WOLINSKI SM, Cancer Cells, 7 (1989) 209. — 15. NOBRE RJ, PEREIRA DE ALMEIDA L, MARTINS TC, J Clin Virol, 42 (2008) 13. — 16. KAY P, MEEHAN K, WILLIAMSON AL, J Virol Methods, 105 (2002) 159. — 17. NAQVI SH, WAJID S, MITRA AB, J Virol Methods, 117 (2004) 91. — 18. SANTIAGO E, CAMACHO L, JUNQUERA ML, VASQUEZ F, J Clin Virol, 25 (2006) 89. — 19. ONG CK, CHAN SY, CAMPO MS, FUIJNAGA K, MAVROMARA-NAZOS P, LABROPOULOU V, PFISTER H, TAY SK, TER MEULEN J, VILLA LL, BERNARD HU, J Virol, 67 (1993) 6424. —20. HEINZEL PA, CHAN SY, HO L, O'ÇONNOR M, BALARAM P, CAMPO MS, FUIJNAGA K, KIVIAT N, KUYPERS J, PFISTER H, STEINBERG BM, TAY SK, VILLA LL, BERNARD HU, J Clin Microbiol, 33 (1995) 1746. — 21. ASTORI G, BELTRAME A, PIPAN C, RAPHENON G, BOTTA GA, Intervirology, 42 (1999) 221. — 22. BERUMEN J, ORDONEZ RM, LAZCANO E, SALMERON J, GASLVAN SC, ESTRADA RA, YUNES E, GARCIA-CARRANCA A, GONZALES-LIRA G, DE LA CAMPA AM, J Nat Cancer Institute, 17 (2003) 1325. — 23. CALLEJA-MACIAS IE, KALANTARI M, HUH J. ORTIZ-LOPEZ R, ROJAS-MARTINEZ A, GONZALES-GUERRERO JF, WILLIAMSON AL, HAGMAR B, WILEY DJ, VILLARREAL L, BERNARD HU, BARRERA-SALDANA HA, Virology, 319 (2004) 315. — 24. PEREZ-GALLEGO L, MORENO-BUENO G, SARRIO D, SUAREZ A, GAMALLO C, PALACIOS J, Am J Clin Pathol, 116 (2001) 143. — 25. DEL REFUGIO GONZALES-LOSA M, LAVIADA MIER Y TERAN MA, PUERTO-SOLIS M, GARCIA-CARRANCA A, J Clin Virol, 29 (2004) 95. — 26. SATISH N, ABRAHAM P, PEEDICAYIL A, SRIDHARAN G, SHAJI RV, CHANDY G, Gynecol Oncol, 95 (2004) 363. — 27. GRASSMANN K, WILCZYNSKI SP, COOK N, RAPP B, IFTNER T, Virology, 223 (2001) 185. — 28. BADARACCO G, RIZZO C, MAFERA B, PICHI B, GIANNARELLI D, RAHIMI SS, VIGILI MG, VENUTI A, Oncol Rep, 17 (2007) 931.

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DETEKCIJA MOLEKULARNIH VARIJANTI HUMANIH PAPILOMA VIRUSA TIPOVA 16 I 6 U ŽENA S RAZLIČITIM CITOLOŠKIM PROMJENAMA RFLP ANALIZOM

SAŽETAK

Infekcije uzrokovane humanim papiloma virusima (HPV) su česte te prisutnost istog genotipa visokog rizika u cervikalnim uzorcima može značiti reinfekciju ili perzistenciju. Perzistentna infekcija je najvažniji prediktor za razvoj karcinoma vrata maternice. Cilj ovog istraživanja je validacija dva seta primera: MY09/11 koij amplificiraju dio gena L1 i P1/P2 koji amplificiraju dio gena E1. Produkt amplifikacije s primerima MY09/11 izložen je digestiji sa 6 restrikcijskih enzima (RE), a produkt amplifikacije s P1/P2 s 12 RE. Analizirani su uzorci od 110 žena koje su se liječile u Klinici za ženske bolesti i porode KBC Zagreb. Amplifikacijom s P1/P2 dobiveno je 98 (89,1%) pozitivnih uzoraka, a s MY09/11 94 (85,5%). Sa MY09/11-RFLP tehnikom dobiveno je 17 različitih genotipova, a sa P1/P2-RFLP tehnikom 7. S oba para primera bilo je pozitivno 82 uzorka; 16 uzoraka bilo je pozitivno samo s P1/P2, a 12 samo s MY09/11. HPV 16 je nađen u 39 uzoraka tehnikom MY09/11-RFLP; od toga su P1/P2-RFLP tehnikom nađene 2 varijante (2 različita obrasca razgradnje) koristeći 3 RE (Dde I, Hae III and Eco I). HPV 6 je nađen u 9 uzoraka tehnikom MY09/11-RFLP; od toga su P1/P2-RFLP tehnikom nađene 2 varijante koristeći samo jedan RE (Hinf I). Kombinacijom 2 PCR-RFLP tehnike otkriveni su subtipovi HPV 16 i HPV 6.