Regular paper

Physical state of human papillomavirus type 16 in cervical intraepithelial lesions and cancers determined by two different quantitative real-time PCR methods*

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The aim of this study was to analyse the correlation between a new multiplex qPCR assay and a reference qPCR assay for assessment of the human papillomavirus (HPV16) load and the viral genome status. The study was performed on 100 HPV16 positive samples containing premalignant lesions and carcinomas. HPV16 *E2* and *E6* gene loads were assessed by two PCR methods. The load of *E2* and *E6* was normalized to the cell number by qPCR targeting the RNase P open reading frame. The physical state of the viral genome was determined as a ratio of *E2/E6* copies number per cell. Among 100 samples analysed, there were no statistically significant differences in the *E2* and *E6* viral load evaluated by multiplex qPCR and qPCR, the correlation coefficients were 0.98 and 0.97, respectively. There were 19% of samples with the integrated, 73% with mixed and 8% with episomal state of viral genome detected by multiplex qPCR and 17%, 79%, 4%, respectively, found by qPCR. Prevalence of integrated and episomal forms estimated by multiplex qPCR was higher than the one obtained by qPCR (Chi², $p < 0.0001$), but in samples with premalignant and malignant diagnoses no significant differences were demonstrated regardless of the methods used. Sensitivity and specificity of multiplex qPCR were 93.7% and 100% as compared with qPCR, the positive predictive value was 100%. In summary, the multiplex qPCR assay in respect of HPV16 load and the frequency of viral genome status was shown to be a sensitive and specific reference method. Simultaneous estimation of *E2* and *E6* genes in one reaction tube reduces the cost of testing.

INTRODUCTION

Human papillomaviruses (HPVs) classified in the *Papillomaviridae* family infect skin or mucosal epithelial cells (de Villiers *et al.*, 2013). Among more than 100 types of HPV, at least 13, are known as high risk type and can cause cancer of the cervix, other anogenital organs, or head and neck. Other HPV types with low oncogenic potential cause non-malignant lesions, such as anogenital warts. In most sexually active women, HPV infections are asymptomatic and transient and only a minority of them will develop persistent infection that could eventually cause precursor lesions such as low- or high-grade squamous intraepithelial lesions (LSIL, HSIL) (zur Hausen, 2002, Munoz *et al.*, 2003).

Several factors contribute to HPV persistence and development of cervical cancer: immunosuppression due to infection with HIV or other microorganisms, multiparity, early initiation of sexual life, long-term hormonal contraceptive use, smoking and infections with other sexually transmitted diseases (Faridi *et al.* 2011). However, additional risk markers are needed which can be host- or virus-related factors such as HPV type, integration stage or viral load. Persistence of high risk type of human papillomaviruses, particularly HPV16, leads to integration of the virus into the host genome and then to disease progression. The genome integration of HPV usually disrupts or deletes *E2*, and rarely *E1* open reading frames, which results in the loss of expression of the corresponding gene products. Disruption of these genes also leads to over-expression of the E6 and E7 oncoproteins, since the *E2* gene product can repress activities from the HPV promoter (Schmidt *et al.*, 2005; Howley *et al.*, 2013). This process leads to increased expression of E6 and E7 viral oncoproteins which target the p53 and pRb tumor suppressor proteins, respectively, resulting in loss of cell-cycle control and downregulation of their anti-tumor functions (Dyson *et al.*, 1989, Scheffner *et al.*, 1990). So far, different methods have been used to determine the HPV16 physical status, such as two-dimensional gel electrophoresis, Southern blotting, *in situ* hybridisation, multiple displacement amplification as an isothermal whole genome amplification technique and various types of PCR (Gallo *et al.*, 2003; Hudelist *et al.*, 2004; Evans *et al.*, 2007). Several methods have been introduced based on PCR: PCR or multiplex PCR assay of *E2* region integrity (Das *et al.*, 1992; Szostek *et al.*, 2008), quantitative real-time PCR (qPCR) assay of the ratio of *E2* to *E6/E7* region amplicons (Peitsaro *et al.*, 2002; Mazumder *et al.*, 2011; Ruutu *et al.*, 2008; Biesaga *et al.*, 2012), amplification of papillomavirus oncogene transcripts test (APOT) (Vinokurova *et al.*, 2008). Test

results obtained by different methods have suggested that the HPV16 integration status might be a marker for cervical precancerous lesion progression.

The aim of present study was to apply a new multiplex real-time PCR-based assay (multiplex qPCR) to assess the HPV16 physical status and load in low- and high-grade squamous intraepithelial lesions of the cervix, and cervical cancer. The results obtained by this new method were compared with reference method - qPCR, in which *E2* and *E6* gene loads are assessed individually.

MATERIALS AND METHODS

Materials. The study was performed on a hundred HPV16 positive samples (66 cervical smears and 34 cervical cancer biopsies) confirmed by the INNO-LiPA HPV genotyping assay (Innogenetics). Clinical materials were obtained from women at ages of 20–77 years (mean 45 ± 15) with different diagnoses: low-grade squamous intraepithelial lesions – LSIL (n = 38), high-grade squamous intraepithelial lesions – HSIL (n = 6) and cervical carcinoma (n = 56, FIGO stage I-III). All cancer patients were treated at the Maria Skłodowska-Curie Memorial Cancer Center and Institute of Oncology, Cracow Branch, Poland. The study has been approved by the Ethics Committee of the Jagiellonian University.

All samples were taken after cytology examination but before treatment. Cervical smears were collected into a saline solution and stored frozen at –70°C until processed.

Cervical cancer biopsies were reviewed by a pathologist in order to confirm diagnoses. For DNA isolation, the paraffin-embedded tissue samples were cut into five to eight 5-μm thick sections.

DNA isolation. DNA extraction was carried out from cervical smears using Genomic DNA Prep Plus kit (A&A Biotechnology, Poland), whereas isolation of DNA from paraffin-embedded samples was performed using EX-WAXTM DNA Extraction Kit (Millipore-Chemicon International, USA) according to the manufacturer's instructions.

Quantitative real-time PCR. Amplification was performed using the ABI Prism 7500 Fast Real-Time PCR Systems for HPV16 *E6* and *E2* genes according to Si *et al.* (2005) using primers, probes and qPCR conditions described by Biesaga *et al.* (2012).

The reference method (qPCR) and multiplex qPCR were performed simultaneously. Quantity of *E2* and *E6* genes was determined separately in qPCR and in one tube in multiplex qPCR.

To generate standard curves for *E6* and *E2* of HPV16, the serially diluted DNA plasmid (pBR322-HPV-16, ATCC 45113) containing 5×10^4, 5×10^5, 5×10^6, 5×10^7, 5×10^8, 5×10^9 HPV-16 DNA genome copy numbers was used in three replicates for each dilution point. Experiments were repeated five times to determine the linearity of amplification. Standard curves were drawn using serial dilutions of known target gene copy numbers vs. corresponding Ct values and fitted by the least-square fit method. SiHa cells were used as positive control of HPV16 integration to host DNA. For *E2* and *E6* gene quantification, the absolute copy number in the unknown samples was calculated by plotting the Ct values against the logarithm of the standard curve.

Each cervical sample was subjected to qPCR for quantification of human RNase P gene. The standard curve used for the human RNase P gene quantification was made according to the manufacturer's instructions

with four decimal serial dilutions of calibrated human genomic DNA, containing 7500, 750, 75, 7.5 RNase P gene copies (TaqMan RNase P Detection Reagents Kit, Applied Biosystems).

Viral load detection. The load of *E2* and *E6* was determined using a copy number normalized per cell. The number of cells was assessed by qPCR targeting the RNase P open reading frame.

Physical state determination. The viral physical state was estimated as a ratio of *E2* to *E6* copy numbers per cell.

To assess the validity of the method, E2/E6 ratios were determined for mixes containing known *E2* and *E6* copy numbers. For this purpose, we prepared a series made with mixes of pBR322–HPV16 plasmids (as episomal forms) and DNA of SiHa cells (as integrated forms; one to two copies/cell) with ratios varying from 0 to 1. All these combinations were analysed in duplicate, and five times independently using the multiplex qPCR method and the reference method — qPCR. The E2/E6 ratios were calculated to determine the pure episomal ($E2/E6 \geq 1.0$), the mixed (both integrated and episomal forms present in a single sample) ($0.99 > E2/E6 > 0.05$) and the integrated ($E2/E6 \leq 0.05$) forms. After this validation step, the DNA from the 44 HPV16 positive premalignant samples and 56 HPV16 positive cervical carcinomas were examined in triplicate by multiplex qPCR and the reference qPCR methods.

Statistical analysis. The statistical analysis was done using the STATA 10.0 software package. Descriptive statistics were used to determine mean and median values of continuous variables and standard errors of means. Correlations between Ct levels were analysed by correlation matrix in which R coefficients and *p* values were calculated. Additionally, statistical significance of the differences between qPCR and multiplex qPCR was established by Student's *t*-test and non-parametric U Mann -Whitney and Kruskal-Wallis tests. Dichotomous variables were analysed using the chi-square test. A *p*-value less than 0.05 was considered significant.

RESULTS

In a first stage of our study standard curves for *E2* and *E6* genes of HPV16 were established. Results estimated in qPCR and multiplex qPCR plotted by least-square fit method were shown in Fig. 1. The sensitivity of both PCR methods was 10^2 viral copies.

Among 100 analysed samples, there were no statistically significant differences in the *E2* and *E6* viral load obtained in the two tested methods (U Mann-Whitney test, p = 0.41 and p=0.34, respectively). The correlation coefficients were 0.98 for *E2* and 0.97 for *E6* gene (Fig. 2).

Physical status of HPV16 genome estimated by multiplex qPCR revealed 19% of samples with integrated, 73% with mixed and 8% with episomal state of HPV16 genome while in qPCR the percentages were 17%, 79% and 4%, respectively. The multiplex qPCR method showed a higher, statistically significant incidence of integrated and episomal forms of viral genome than this obtained by qPCR (Chi², *p* < 0.0001). These results are shown in Fig. 3. Viral load of HPV- (targeting HPV16 *E6* open reading frame) for each physical stage estimated in both methods is presented in Fig. 4. The prevalence of integrated, episomal and mixed forms of HPV16 genome in samples with premalignant and cervical cancer diagnoses did not significantly differ in both of the stud-
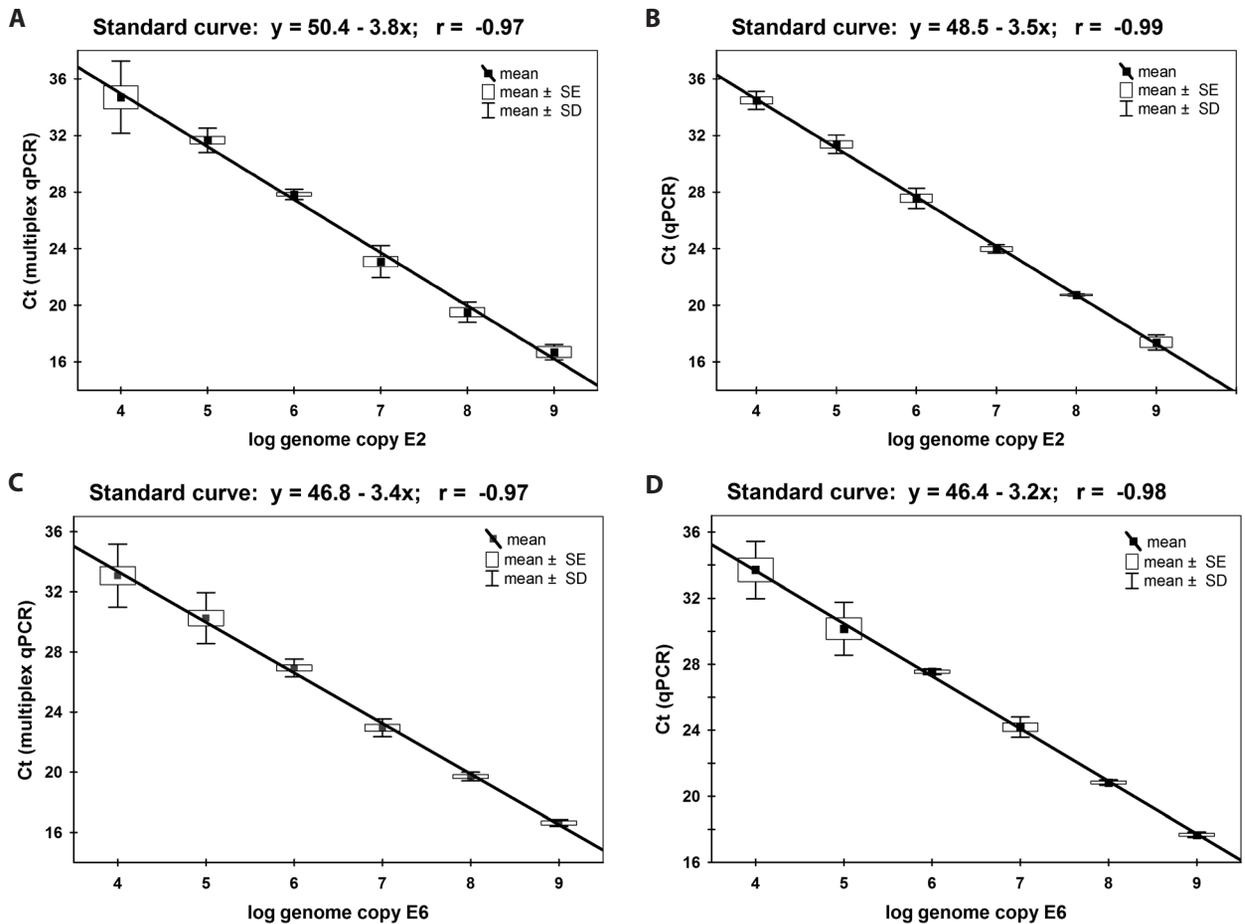
A

Standard curve: y = 50.4 - 3.8x; r = -0.97

B

Standard curve: y = 48.5 - 3.5x; r = -0.99

C

Standard curve: y = 46.8 - 3.4x; r = -0.97

D

Standard curve: y = 46.4 - 3.2x; r = -0.98

Figure 1. Standard curves for HPV-16 E2 and E6 sequences tested by multiplex qPCR (A, C) and qPCR (B, D).

logE2 multiplex qPCR = -0.0090 + 0.96354 * logE2 qPCR
correlation: r = 0.94

logE6 multiplex qPCR = 0.02686 +0.95960 * logE6 qPCR
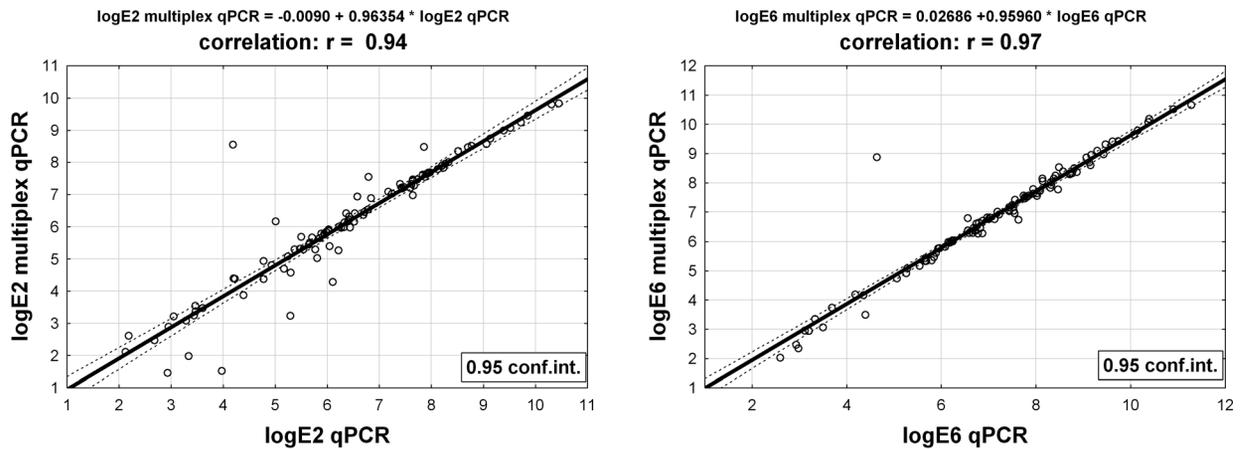**correlation: r = 0.97**

Figure 2. Correlation between results obtained by multiplex qPCR and qPCR for the HPV-16 E2 and E6 sequences in 100 samples analyzed.

ied methods (Pearson's Chi^2 test, $p=0.2$) (Fig. 5). The sensitivity and specificity of multiplex qPCR were 93.7% and 100% as compared to qPCR, positive predictive value was 100%.

DISCUSSION

It is well-known that a high-risk HPV infection may progress to cervical cancer within several years, but only about ten percent of these infections persist in host cells and evolve into premalignant lesions (Moscicki *et al.*, 2006). Therefore, it is important to identify the biomarkers that contribute to the development of cervical cancer and then determine women with a real risk of cancer. The HPV16 DNA integration into host genome has been regarded to be a key step in the progression towards invasive cancer (zur Hausen, 2009). However, the frequency of viral integration into the host genome in premalignant and malignant lesions is under discussion (Nagao *et al.*, 2002; Peitsaro *et al.*, 2002; Hudelist *et al.*,

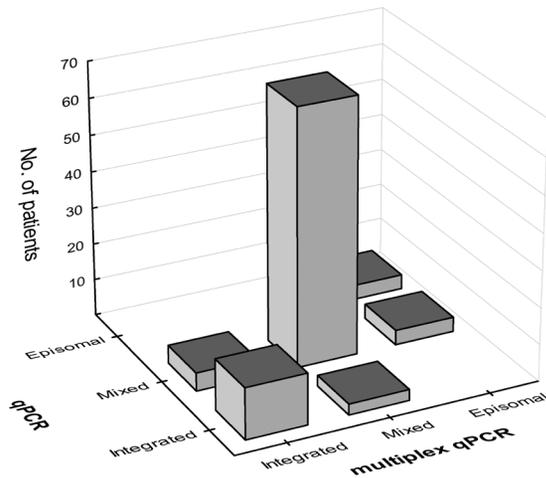**multiplex qPCR:qPCR: Chi^2 = 100,5697; p = 0.0000; N = 100**

Figure 3. The physical status of HPV-16 genome estimated using multiplex qPCR and qPCR.

Figure 4. Comparison of HPV-16 viral load in different physical statuses of genome.
Differences are statistically significant ($p < 0.001$, Kruskal-Wallis test).

2004; Kumala *et al.*, 2006; Arias-Pulido *et al.*, 2006; Shukla *et al.*, 2014; Ribeiro *et al.*, 2014). It has been proposed that integration can either be an early event associated with progression from LSIL to HSIL, or a later event that accompanies progression from HSIL to cervical cancer (Groves & Coleman, 2015).

The main methods for detection of HPV integration are based on fluorescence *in situ* hybridization, PCR, and qPCR (Abreu *et al.*, 2012). In the present study, the ratio between the levels of viral *E2* and *E6* loads was calculated by a new multiplex qPCR assay and qPCR as the reference method.

In our study, criteria for assessing the HPV-16 physical status were estimated based on artificial combination of DNA isolated from SiHa cells and pBR322-HPV-16 plasmid. The established values for each status ($E2/E6 \geq 1.0$ for pure episomal, ≤ 0.05 for integrated and between 0.05–0.99 for mixed (integrated and episomal forms) were similar to those of Mazumder Indra *et al.* (2011) and Dutta *et al.* (2015), who used Power SYBR
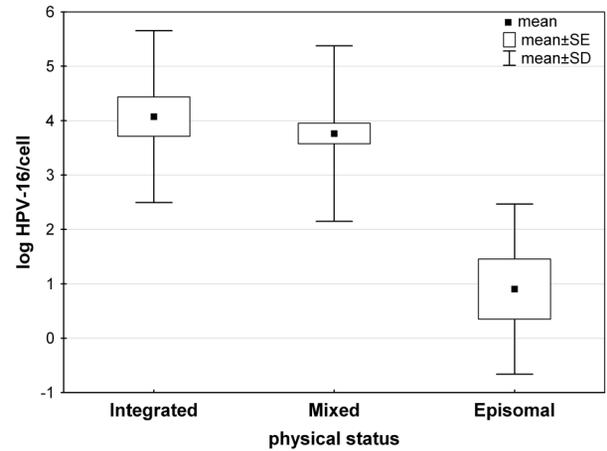
Green qPCR for HPV16 physical state assessment. Other authors have used a slightly different criteria and the differences were mostly related to the cut-off for the episomal form (Peitsaro *et al.*, 2002; Arias-Paulido *et al.*, 2006; Cricca *et al.* 2007; Saunier *et al.*, 2008;). The ratio of $E2/E6 \geq 1.0$ for the pure episomal form was used the most frequently (Kulmala *et al.*, 2006; Mazumder Indra *et al.*, 2011; Biesaga *et al.*, 2012; Shukla *et al.*, 2014). Canadas *et al.* (2010a) applied a multiplex qPCR based on validation of simultaneous amplification of *E2* and *E6* HPV16 genes in SiHa cells using as a control for HPV16 integration and anal cell samples from asymptomatic patients with transient HPV16 infection as an episomal control. In subsequent studies, this method was used for the detection of HPV16 physical status in cervical exfoliated cells obtained from women with normal and atypical cytology and cervical cancer (Canadas *et al.*, 2010b; Ribeiro *et al.*, 2014). Based on these studies and material with different severity of the lesions, these authors confirmed that the integration of HPV16 may be a good biomarker for the evolution of CIN into cervical carcinoma but further research is required to corroborate it. Other authors also drew attention to the direct cor-
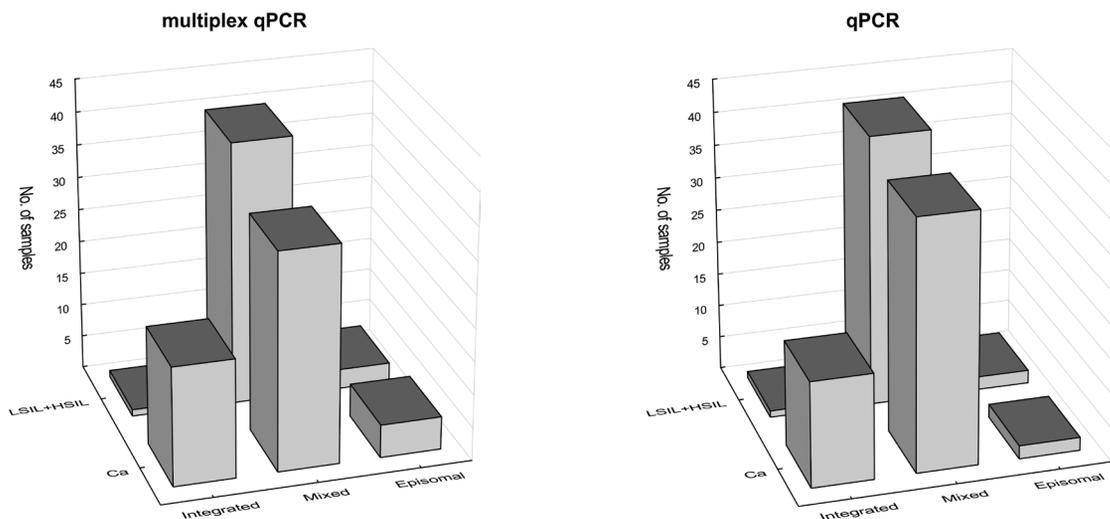

Figure 5. Comparison of HPV-16 physical status in premalignant and cervical cancer samples estimated by multiplex qPCR and qPCR.

relation between the frequency of integration of HPV16, which increased with the severity of lesions in the cervix (Guo et al., 2007; Saunier et al., 2008; Shukla et al., 2014). In our study, women with invasive cervical cancer accounted for 56% of the studied population and the HPV16 integration in this group was present only in 29% and 32% of cases, depending on the method applied (multiplex qPCR and qPCR, respectively). In the subgroup of women with a premalignant lesion, the viral integration was confirmed only in one case. In most women with cervical cancer, the mixed form of the genome was detected (59% and 68% by multiplex qPCR and qPCR, respectively). Such a high frequency of the mixed form in cancer patients may result from the fact that excess episomal copies might mask the integrated form of HPV16 (Ruutu, 2008). On the other hand, it may be a consequence of differences in FIGO stage among studied cancers. Arias-Pulido et al. (2006) showed significant differences in the occurrence of the mixed form of HPV16 regarding carcinoma in situ and invasive cervical cancer, in 29.4% and 47.5%, respectively. The high rate of the mixed form of HPV-16 in our samples may also be explained by the presence of a mutation in the E2 sequence or the viral DNA disruption outside the E2 region (Arias-Pulido et al., 2006; Jiang et al., 2009; Li et al., 2013).

Mixed form of the viral genome was detected as the most common in premalignant lesions (41%) and cervical cancer (68%). This result is similar to the one demonstrated by other authors using qPCR (Kulmala et al., 2006; Guo et al., 2007; Saunier et al., 2008).

In our cases, the frequency of integrated form correlated in a statistically significant way with HPV16 load. Viral load increases with severity of lesions. To our knowledge, there are no published studies in which the results achieved by qPCR and multiplex qPCR are compared. Only in the discussion section of the paper by Peisaro et al. (2002), the authors' preliminary experiments with multiplexing qPCR were mentioned but as quoted "the data obtained were less reliable than those obtained when the reaction was ran in two separate tubes". In conclusion, our results obtained by multiplex qPCR in respect of HPV16 load and the frequency of viral genome status are similar to those obtained by the reference method. Since multiplex qPCR allows for simultaneous evaluation of the two HPV genes in one reaction tube and thereby for reduction of the cost, we recommend this test for cervical cancer screening. Presented results suggest that HPV16 viral load and physical status of viral genome have predictive potential as biomarkers of carcinogenesis, although further studies are needed to confirm this thesis.

Acknowledgements

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