The HNSCCs is the sixth leading cancer by incidence worldwide and eighth by death. There are 0.5 million new cases a year worldwide [5]. The environmental pollution and wrong lifestyle, which are associated with the squamous cell carcinomas, involve following risk factors: tobacco smoking, alcohol consumption, UV light, particular chemicals used in certain workplaces, and certain strains of viruses, such as human papillomavirus (HPV) [6].

Human papillomaviruses (HPVs) are small DNA viruses with a strict tropism for human epithelial cells. In general, HPV infections are asymptomatic and persist for 18–24 months before they are cleared by the immune system of the host [7]. Tumorigenicity of HPVs differs markedly among HPV genotypes [8]. This virus...
has been associated with HNSCCs in the areas of the oropharynx, lung, fingers, and anogenital region [1]. Functional loss of p53 tumor suppressor gene, mutations in Ras protooncogenes, and certain chromosomal aberrations are characteristics of cancer caused by HPVs [9].

Many different methods are able to give information about the presence of HPVs in biological samples. The combination of a sensitive test, and a specific test, could allow for the best potential to accurately establish the presence or absence of HPVs [10,11]. The mainly utilized tests for HPV identification among others, are polymerase chain reaction (PCR) testing, real-time PCR, in situ hybridization analysis (ISH), immunohistochemical (IHC) staining for tumor suppressor protein p16, and southern blotting assays [11].

The methodology for polymerase chain reaction (PCR) is an indispensable technique used in molecular biology in research labs to amplify a single copy of DNA generating thousands to millions of copies of chosen DNA sequence [12]. This technique has a big variety of applications like DNA cloning for DNA sequencing, DNA-based phylogeny, or functional analysis of genes; the diagnosis of hereditary diseases; the identification of genetic fingerprints and the detection and diagnosis of infectious diseases. PCR can be extensively modified to perform a wide array of genetic manipulations [10,13].

In HPV detection the PCR represents a highly-sensitive and cost-effective method. It can be used to detect as little as one copy of a DNA sequence from paraffin-embedded tissues or fresh tissues from biopsies [14]. The disadvantages of PCR techniques are that they have lower specificity, they do not allow distinction between all HPV types that are present in the neoplastic cells and non-neoplastic and they cannot distinguish between episomal and integrated HPV DNA [15]. Furthermore, the presence of latent viruses leads to false positive results due to the ability of PCR to detect just a few copies of HPV DNA per cell. Different attempts have been made to resolve this issue through the use of real-time PCR, which provides a quantitative analysis of viral load [16].

The combined results of immunocytochemical detection of p16 protein and the detection of HPV DNA by specific PCR enable better discrimination between latent and carcinogenic HPV infections and thus can provide information on the prognosis of HNSCC patients and facilitate therapeutic decisions [17].

The detection of the viral oncoproteins E6 and E7 requires technique that is restricted to the research laboratory, like RNA extraction and polymerase chain reaction amplification. The development of RNA in situ hybridization (ISH) probes complementary to E6/E7 mRNA permits direct visualization of viral transcripts in routinely processed tissues and has opened the door for accurate HNSCC detection in the clinical care setting [18].

### Table 1: Clinical characterization of patients.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Age</th>
<th>Histological group</th>
<th>Localization</th>
<th>Grade</th>
<th>Stage</th>
<th>Metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>92% Men</td>
<td>69</td>
<td>97% Spinocellular</td>
<td>73% Dissem</td>
<td>95% High</td>
<td>49% I-II</td>
<td>5%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>51% III-IV</td>
<td></td>
</tr>
<tr>
<td>8% Women</td>
<td>64</td>
<td>99% Spinocellular</td>
<td>81% Dissem</td>
<td>93% High</td>
<td>69% I-II</td>
<td>6%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>51% III-IV</td>
<td></td>
</tr>
</tbody>
</table>

2. Results and Discussion

2.1 Clinical Characterization of Patients

In this study 103 DNA samples were isolated from leukocytes of patients with head and neck cancer from St. Anne’s University Hospital in Brno and purified using QIAamp DNA Mini Kit. The average age of the patients was 69 years for
men and 64 years for women.

92% of the patients were men and 8% women, with major diagnosis for both which was oropharynx cancer with spinocellular histological type, followed with adenocarcinomas and basaliomas. Only a low percentage of the high grade tumors in men and women were found. These men presented a 51% of this cancer in the stage III-IV, with a 5% of metastasis, while the women presented a 69% of this cancer in the stage I-II, with a 6% of metastasis (Table.1).

2.2 PCR detection and subtypes identification

The samples were analyzed using PCR with specific primers for HPV detection and subtypes identification. DNA from leukocytes samples was tested using GP5/GP6 set of primers located within the L1 region of HPV genome. Furthermore, the samples were also tested with type-specific primers for HPV types 16 and 18 (Fig. 1). The quality of the isolated DNA, was determined with β-actin gene. 85 of the 103 DNA samples were β-actin positive and thus, adequate for further analysis. In this group of samples, 56 positives samples were detected for HPV which were obtained in 65.9% cases. We identified the subtypes with type-specific primers for HPV-16 and HPV-18, obtaining 12 samples of HPV-16, 7 samples of HPV-18 and 4 samples were HPV-16 and HPV-18 at the same time. (Fig. 1). The HPV positive samples were also detected by PCR sequencing. After purification and sequencing of these samples, they were studied by in silico analysis using BLAST searches and Clustalw Multiple Sequence Alignment, obtaining 73% of identities with full length sequence of L1 HPV major capsid protein (Fig.3).

2.3 Sequencing analysis

The HPV positive samples were also checked by PCR sequencing. After purification and sequencing of these samples, they were studied by in silico analysis using BLAST searches and Clustalw Multiple Sequence Alignment, obtaining 73% of identities with full length sequence of L1 HPV major capsid protein (Fig.3).

2.4 Discussion

We have used in our study the polymerase chain reaction (PCR) method for HNSCCs detection in leukocyte samples, because is a highly-sensitive, fast and cost-effective method. The PCR methods enable the detection of large number of HPV genotypes and also can increase significantly the positivity rate of HPV DNA detection in samples with a low DNA copy number. In conclusion, we preselected the samples using sets of primers located within the L1 region of HPV genome (GP5/GP6 primers) and with an additional amplification to typing of PCR-positive samples with the HPV type-specific primers (HPV16-18 primers). The samples were also tested by PCR sequencing and by in silico analysis, where was obtained a 73% of identities with the sequence of L1 HPV major capsid protein, which confirms the presence of HPV.

The first studies that used the set of GP5/GP6 primers for HPV detection were done in samples of patients with cervical cancer. Jacobs et al., introduced a GP5/GP6 PCR-based procedure in which they included Southern blot
hybridization of PCR products with cocktails of radioactively labeled HPV type-specific internal oligonucleotides to detect a broad spectrum of genital HPV genotypes in one PCR[19]. De Roda Husman et al observed that the GP5/GP6 PCR method provided an increased detection of HPV level in poorly cervical scrapes[20]. Baay et al, compared the efficacies of three general primer pairs (GP5/6, CPI/IIG, MY09/11) for the detection of HPV DNA in formaldehyde-fixed paraffin-embedded carcinomas. The efficacy of each primer pair increased in HPV DNA detection in a 87.6%[21].

3. Experimental Section
3.1 Clinical specimens
A total of 103 histopathologically confirmed head and neck cancer subjects were involved in the study. The samples were collected from St. Anne’s University Hospital, Department of Otorhinolaryngology and Head and Neck Surgery. Enrolment of patients into realized clinical study was approved by the Ethic Committee of the Faculty of Medicine, Masaryk University, Brno, Czech Republic.
3.2 Extraction and quantification of DNA

DNA was isolated and purified from leukocytes using QIAamp DNA Mini Kit (Qiagen, Venlo, Netherlands), following the manufacturer’s instructions. The concentration of purified DNA was determined using multimode reader Infinite 200 (Tecan, Männedorf, Switzerland).

3.3 Polymerase chain reaction (PCR)

The 142 base-pair long sequence of L1 gene was amplified using GP5 and GP6 primers. The PCR mixture from New England Biolabs (UK), containing the PCR buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl with 2.5 mM MgCl₂ included) 0.05 mM of each dNTP and 0.05 mM of GP5 and GP6 primers (Table 2). The DNA amplification was carried out during 40 cycles that included the denaturation at 94°C for 30 s, the annealing at 58°C for 30 s and the primer extension at 72°C for 30 s.

As the internal quality control of the isolated DNA, β-actin gene (600bp) was used. The PCR mixture from New England Biolabs (UK), contained the PCR buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl with 2.5 mM MgCl₂ included) 0.05 mM of each dNTP and 0.05 mM of each couples of primers (Table 2). The DNA amplification was carried out during 40 cycles that included the denaturation at 94°C for 30 s, the annealing at 58°C for 30 s and the primer extension at 72°C for 45 s. Each PCR product was analysed using electrophoresis on 1% agarose gels stained with ethidium bromide.

3.4 Sequencing

GenomeLab DTCS Quick Start kit (Beckman Coulter, USA) with 20 ng of purified amplified DNA from the PCRs was used for the sequencing reaction. The cycling conditions were as follows: 30 cycles of denaturation at 96 °C for 20 s; annealing at 50 °C for 20 s and 60 °C for DNA synthesis for 4 min. DNA fragments from this reaction were purified using magnetic particles CleanSEQ (Beckman Coulter, USA). DNA sequencing was performed on Genetic Analysis System CEQ 8000 (Beckman Coulter, USA). After denaturation at 90 °C for 2 min, the fluorescence-marked DNA fragments were separated in 33 cm capillary with 75 μm i.d.

### Table 2: List of primers used in this study [25].

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GP5</td>
<td>TTTGTACTGTTAGATAC</td>
<td>(Snijders et al., 1990[22])</td>
</tr>
<tr>
<td>GP6</td>
<td>GAAATAAATGTAAATCA</td>
<td></td>
</tr>
<tr>
<td>HPV16fw</td>
<td>CCCAGCTGTAATCATGCATGGAGA</td>
<td>(Soler et al., 1991[23])</td>
</tr>
<tr>
<td>HPV16rv</td>
<td>GTGTGCCCAATACAGGTTCTTCCA</td>
<td></td>
</tr>
<tr>
<td>HPV18fw</td>
<td>CGACAGGAACGACTACAGCA</td>
<td>(Soler et al., 1991[23])</td>
</tr>
<tr>
<td>HPV18rv</td>
<td>GCTGCTAATTGATGATTACT</td>
<td></td>
</tr>
<tr>
<td>β-actin fw</td>
<td>CCTGAACCCCTAAGGCCAACC</td>
<td>(Ueyama et al., 1987[24])</td>
</tr>
<tr>
<td>β-actin rv</td>
<td>GCAATGCTGGGTACATGGT</td>
<td></td>
</tr>
</tbody>
</table>
(Beckman Coulter, USA), which was filled with a linear polyacrylamide denaturing gel (Beckman Coulter, USA). The separation was performed at capillary temperature of 50 °C and voltage of 4.2 kV for 85 min.

3.5 Sequence analyses
Sequence analyses were performed using, BLASTX algorithm available from the NCBI (http://blast.ncbi.nlm.nih.gov/;[26]) and ClustalW2 algorithm available at EBI (http://www.ebi.ac.uk/Tools/clustalw2/;[27]).

4. Conclusions
The polymerase chain reaction is a suitable method enabling the detection of HPVds and differentiation between different subtypes. Many different HPV tests are available. All of the methods are able to give information about the presence of HPV in biological samples. The majority of carcinomas are caused by the most common and high risk HPV types, HPV-16 and HPV-18, which contain the E6-E7 oncogenes. The patients infected with one type of HPV were significantly more likely to harbor additional HPV types. Thus, evaluation of risk rate of heterogeneous coinfections has to be further done to determine the effects on head and neck cancer development.

Acknowledgments
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Conflicts of Interest
The authors declare they have no potential conflicts of interests concerning drugs, products, services or another research outputs in this study. The Editorial Board declares that the manuscript met the ICMJE „uniform requirements“ for biomedical papers.

References


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