HUMAN PAPILLOMAVIRUS INFECTION AMONG WOMEN WITH NORMAL CERVICAL PAP SMEAR TESTS

P. Draganov¹, D. Georgiev², A. Gancheva³, M. Sayej¹, Z. Kalvatchev¹
National Center of Infectious and Parasitic Diseases, Laboratory of Molecular Virology, Sofia, Bulgaria¹
Center for Modern Obstetrics and Gynecology Aid, Sofia, Bulgaria²
Specialized Hospital for Active Treatment of Oncologic Diseases, Sofia, Bulgaria³

ABSTRACT
Various human papillomaviruses (HPVs) have been strongly implicated as etiological agents in human cervical cancer. The primary method for detection of high-risk HPVs is still the Papanicolaou-stained (Pap) smear. However, the Pap smear has some limitations, including high false-negative rates. Using consensus and specific primer-mediated polymerase chain reaction (PCR), we investigated 55 women with normal cervical cytology. HPV DNA was found in 11 women (20%), including HPV-16 in 6 cases (10.9%), HPV-6 in 3 cases (5.5%), HPV-6/18 in 1 case (1.8%) and HPV-6/33 in 1 case (1.8%). The obtained results demonstrate the importance of careful follow-up and repeated testing for HPV persistence. Moreover, these findings raise the possibility that HPV diagnostics by molecular virology techniques might be useful as an adjunct to Pap smear screening.

Introduction
Human papillomavirus (HPV) is the principal risk factor associated with cervical cancer, one of the most common malignancies among women. Based on their association with cervical cancer and precursor lesions, HPVs can be divided into high-risk or oncogenic types (e.g. HPV-16, -18) and low-risk or non-oncogenic types HPV types (e.g. HPV-6, -11) (2).

The main approach for identification of cell changes in the cervix is cytology. Papanicolaou-stained (Pap) smear is easy to obtain and has relatively low cost, it is an excellent screening test. Despite its success, the Pap smear has some limitations. Inadequate samples constitute about 8% of specimens received (8). False-negative rates as high as 20 to 30% have been reported (8). Besides, cytology can ensure only indirect data about HPV presence. The advent of molecular diagnostic techniques has led to rapid advances in HPV diagnostic capabilities. HPV diagnosis based on amplification of segments of the viral genome by polymerase chain reaction (PCR) combines sensitivity and specificity (8). By means of molecular techniques it is possible to perform the detection of viral genomic DNA and identification of HPV types.

In this report we describe our findings regarding general and type specific HPV prevalence in 55 women with normal Pap smear tests (cytologically normal women), using a multiplex PCR for detection of specific viral DNA in cervical cells.

Materials and Methods
Patients and samples. Fifty-five women aged between 20 and 40 years, living in Sofia were included in the study. All women were tested cytologically and found to be normal. Samples of cervical cells
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taken by cytobrush were obtained from each woman and were resuspended in transport buffer (5). Total cellular DNA was extracted with DNAzol (Invitrogen, USA) or by standard phenol-chloroform procedure (2). The extracted DNA was analyzed immediately or stored at –70°C until use.

**HPV detection and typing.** The cervical DNA was tested for the presence of specific HPV DNA fragments, using the previously adapted in our laboratory PCR techniques (5). The amplification reaction included consensus primer pair MY09/MY11, amplifying a 450-bp fragment in the L1 open reading frame of numerous HPV types. To control internally the quality of the isolated DNA, the 110 bp sequence of β-globin gene was co-amplified using PC03 and PC04 primers in the multiplex PCR with the MY primers. Than, all HPV positive samples were subjected to specific primer-mediated PCR. Type-specific primers for HPV-6, -11, -16, -18, -31, -33 were used (5). Positive and negative controls were included to verify the results and to avoid false-positive signals due to contamination. Aliquots (20 ul) of each product were resolved by electrophoresis in a 2% agarose gel and stained with ethidium bromide.

**Results and Discussion**

All laboratory prepared cellular DNA samples were suitable for investigation. Eleven (20%) of eligible 55 women were found to be positive for HPV presence. Selected amplicons obtained after the consensus primer-mediated HPV-PCR for simultaneous detection of L1 and β-globin genes are shown on the Fig. 1.

**Table 1** presents specific HPV type distribution among the infected women. Both mono- (HPV–6 or –16) and double-infections (HPV–6/18 or –6/33) were observed. As the age is concerned, all the infected women were < 30 years of age (Table 2).

It is known, that cervical HPV infection is common among young sexually active women (1, 4, 10). The number of the tested women in our study is relatively small. Nevertheless, the overall HPV prevalence was 20%, which is in accordance with the studies performed in various parts of the world, including Bulgaria (3, 6, 7, 8, 9). We are planning to expand such a group in future and investigate the eventual viral persistence.

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**FIG. 1.** Multiplex PCR for consensus HPV detection. Lane 1: HPV positive control; Lane 2: HPV negative control; Lane 3: HPV negative cervical specimen; Lanes 4 – 7: HPV positive cervical specimens; Lane M: DNA MW marker.

**TABLE 1**

<table>
<thead>
<tr>
<th>HPV type</th>
<th>Positive cases</th>
</tr>
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<tbody>
<tr>
<td>6</td>
<td>3 (5.5%)</td>
</tr>
<tr>
<td>16</td>
<td>6 (10.9%)</td>
</tr>
<tr>
<td>6 + 18</td>
<td>1 (1.8%)</td>
</tr>
<tr>
<td>6 + 33</td>
<td>1 (1.8%)</td>
</tr>
</tbody>
</table>

**TABLE 2**

<table>
<thead>
<tr>
<th>Patient age</th>
<th>Number of analyzed specimens</th>
<th>HPV positive specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 – 24</td>
<td>15</td>
<td>6</td>
</tr>
<tr>
<td>25 – 29</td>
<td>14</td>
<td>5</td>
</tr>
<tr>
<td>30 – 34</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>35 – 39</td>
<td>14</td>
<td>0</td>
</tr>
</tbody>
</table>
The results of our study suggest that both high-risk HPV types, and low-risk types are prevalent among the studied women. High-risk HPV-16 was found in 10.9% of the tested women; low-risk HPV-6 appeared in 5.5% of cases. HPV-16 seems to be the most prevalent HPV type among cytologically normal women (6). However, the proportion of the women found to be HPV-16 positive varies throughout the world – 84% for England, 33% for Australia, 16% for Japan and 6.9% for Greece (6). The discrepancies are probably related to different sexual habits, to different incidence of each HPV type in various countries, or to differences in analytical procedures. Two double infections were also detected in our study, the first was HPV-6/18, and the second was HPV6/33. According to Morrison E. et al., 1994 (8), infection with multiple HPV types has been associated with an increased risk of cancer precursors, however it is not known whether specific HPV types could promote or exclude infection with specific other HPV types.

We observed that HPV prevalence was relatively high among women younger than 30 years and then declined sharply with increasing age. Several investigators have found the presence of HPV to be distinctly uncommon in cytologically normal women who are >35 years of age (6, 7, 8). These studies have been conducted in geographically different populations and with use of different methodologies. It has been suggested that infection frequently occurs within several years of the onset of sexual activity, running self-limiting course in individuals who ultimately develop neoplastic disease. This result has been interpreted as an indicator of the sexual transmission as it coincides with initiation of sexual activity (8).

In conclusion, HPV prevalence in the tested women with normal Pap smear tests is relatively high. As the persistence of high-risk HPV is a significant risk factor for the development of proliferative lesions and their progression, HPV-positive cases should be subjected to a careful follow-up and repeated HPV testing. The obtained results raise the possibility that HPV diagnostics by molecular virology techniques might be useful as an adjunct to Pap smear screening.

REFERENCES