

**Short
Communication****Human papillomavirus (HPV) study of 2916
cytological samples by PCR and DNA sequencing:
genotype spectrum of patients from the west
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Human papillomaviruses (HPVs) are aetiological agents for cervical cancer. More than 70 different HPV types that infect genital mucosa have been found. In order to develop a sensitive and specific detection and typing assay, a PCR/direct sequencing approach was used. Two pairs of consensus primers were used for amplification of HPV DNA and the PCR products obtained were analysed by automated sequencing. Sequences were compared with those in GenBank by using the BLAST program. In this study, 2916 cytological samples were screened for HPV, as well as for triage. Nine hundred and forty-eight (32.5%) samples were positive for HPV, of which 134 harboured more than one HPV type. Of the 948 PCR-positive samples, 648 were typed. Thirty-nine different HPV types were identified by sequencing. The two most frequently found HPV types, 16 and 31, together accounted for 36.3% of the sequences (26.2 and 10.1%, respectively). This group was followed by HPV types 6 (5.7%), 18 (5.3%), 58 (4.5%), 61 (4.5%), 53 (4.4%), 42 (4.3%) and 51 (4.0%). All other types were detected at frequencies <4% and eight types were detected only once. PCR/direct sequencing is a reliable method for routine detection of HPV in cytological samples. The data presented here suggest a complex distribution of HPV types in the population tested. The results accentuate the importance of PCR-based techniques in HPV diagnosis, as hybridization-based methods can only detect a limited number of infections. This method can also be applied easily to the analysis of tissue samples and it therefore also allows type-specific follow-up of women who have been treated for cervical intraepithelial neoplasia.

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Introduction

Cervical cancer is the second most common cause of cancer-related death in women. Specific anogenital types of human papillomavirus (HPV) cause the initiating infection that leads to cervical cancer. More than 100 HPV types are known, of which at least 70 infect the anogenital tract. Knowledge of HPV status is becoming increasingly important as a triage screen after detection of atypical cells of undetermined significance (Bollmann *et al.*, 2003b) and as a primary screen for cervical cancer detection (Cuzick, 2000). HPVs are classified into low- and high-risk categories, based on their association with malignant lesions and phylogenetic relationships (Lorincz *et al.*, 1992; Walboomers *et al.*, 1999). Therefore, HPV typing has an important prognostic or

therapeutic value, as it can distinguish between HPV types of high and low oncogenic risks. Identification of high-risk HPV genotypes may permit selection of those patients who are at increased risk for disease and may therefore provide additional clinical value. An important requirement for this approach is that HPV testing and identification of high-risk HPV types should be highly sensitive and specific.

The commercially available hybrid capture (HCII) assay is used widely in routine analysis of cervical scrapings, but does not allow typing of viruses. This test also permits the detection of only the limited number of genotypes that are included in the hybridization probe mixtures. Therefore, it cannot detect relatively rare or novel HPV types.

In contrast to the HCII assay, consensus PCRs have been devised to amplify and detect all HPV types. The majority of large HPV-testing studies have been performed with the MY09/MY11 and GP5+/GP6+ primer sets. Analysis and typing of PCR products were done routinely by type-specific

Abbreviations: CIN, cervical intraepithelial neoplasia; HCII assay, hybrid capture assay; HPV, human papillomavirus; HSIL, high-grade intraepithelial lesions; SIL, squamous intraepithelial lesions.

oligonucleotide hybridizations or by real-time fluorescence PCR assays that used molecular beacons for HPV typing (Szuhai *et al.*, 2001). We used both of the consensus primers for routine PCR testing of HPV, in combination with automated PCR fragment analysis and PCR/direct sequencing.

Here, we report a sequenced-based HPV study of 2916 liquid-based cytological samples from a west German area (North Rhine–Westphalia). It allows an insight into the complex distribution of HPV types in the population tested.

Methods

Source of specimens. Cervical samples of 2916 women (median age, 40.5 years; range, 17–83 years) from the west German area (North Rhine–Westphalia) were tested for HPV in this study. Routine liquid-based cervical cytological samples in PreservCyt solution (Cytic), which were collected by private gynaecologists, were used. Samples were screened for HPV in the absence of clinical signs of cervical dysplasia ($n = 1720$; 59%) and for the confirmation and risk classification (triage) of cervical dysplasia ($n = 1196$; 41%). Cytological screening of the samples was done by trained cytologists.

HPV detection and typing. Samples (10 ml) were centrifuged at 2000 g and the supernatant was removed. An aliquot (200 µl) of this concentrated sample was used for DNA isolation with a QIAamp DNA Mini kit (Qiagen), according to the manufacturer's instructions. DNA was eluted from columns in a volume of 100 µl 10 mM Tris/HCl, pH 8.0.

General consensus primers GP5+/GP6+ (Jacobs *et al.*, 1997) and MY09/MY11 (Bauer *et al.*, 1992) were used to amplify the corresponding part of the HPV L1 gene. Presence of human genomic DNA was verified by amplification of a 268 bp fragment of the β -globin gene by using primers Glob-F (5'-CAACTTCATCCACGTTCCACC-3') and Glob-R (5'-GAAGAGCCAAGGACAGGTAC-3'). Forward primers used in the PCRs were labelled with fluorescent dyes: GP5+, MY11 and Glob-F with HEX, FAM and NED, respectively. PCR conditions were as follows: preheating for 5 min at 94 °C was followed by 40 cycles of 20 s at 94 °C, 20 s at 39 °C (GP5+/GP6+) or 55 °C (MY09/MY11; Glob-F/Glob-R) and 40 s at 72 °C, and a final extension of 5 min at 72 °C. PCRs were performed in a final volume of 20 µl, which consisted of 3 µl extracted DNA as a template, 2 µl 10 × PCR buffer, 1 U Platinum *Taq* polymerase (Invitrogen), 20 pmol each primer and 0.2 mM dNTPs. A MgCl₂ concentration of 1.5 mM was used for all primers. Positive controls were performed with purified DNA from the HPV 16-positive Caski cell line. The number of samples run in one batch ranged between 12 and 24, including positive and negative controls.

An aliquot (0.8 µl) of each of the three PCR products was mixed with 10 µl formamide and 0.5 µl internal standard (GENESCAN 500-ROX; Applied Biosystems). After denaturation at 90 °C for 2 min and cooling, PCR products were analysed by using an automated ABI 310 genetic analyser (Applied Biosystems). PCR product size was determined by using GENESCAN software 2.1.1 (Applied Biosystems). By monitoring the fluorescence intensity of the positive controls in each run and by testing randomly selected samples in duplicate in one run, we found no significant inter- or intra-assay variation.

Positive PCR products were purified by using a High Pure PCR product purification kit (Roche Diagnostics) according to the manufacturer's instructions. PCR products were subsequently sequenced with a Big-Dye Terminator sequencing kit (Applied Biosystems) by using one of the PCR primers as a sequencing primer. Sequencing reactions were also analysed on the ABI 310 genetic analyser (Applied Biosystems).

Obtained sequences were compared with documented virus sequences that were available in GenBank by using the BLAST program (Altschul *et al.*, 1997).

HPV type-specific PCR was performed as described by van den Brule *et al.* (1990) with modifications. All forward primers used in PCRs were labelled with FAM; this allowed analysis of PCR fragments by fluorescence capillary electrophoresis, as described for the consensus PCRs. Type-specific PCRs were only used in the typing of multiple HPV infections.

With a randomly selected group of samples that tested positive for HPV with our method ($n = 50$), we performed the INNO-LiPA line probe assay (Innogenetics) according to the manufacturer's instructions.

Results and Discussion

Among the 2916 samples tested, 32.5% (948) were HPV-positive by PCR, of which 14% (134/948) showed multiple infections (for the most part, double infections). Samples used in this study were not selected randomly, as samples screened for triage after detection of cytological signs of dysplasia were included. Therefore, a relatively high rate of positive HPV test results was obtained.

All samples were tested with both HPV consensus primer systems; PCR results correlated in most cases. Discordant patterns were obtained with HPV types 30, 42, 43, 51, 59, 67, 74, 90 and 91, which were positive with Gp5+/Gp6+ and negative with MY09/MY11. In contrast, HPV types 61 and 62 were only detected by the MY09/MY11 primers.

The test system used has 98.2% sensitivity in the identification of women with squamous intraepithelial lesions (SIL). Patients with high-grade intraepithelial lesions (HSIL) were all detected (Bollmann *et al.*, 2003a).

Our method allowed us to detect and type multiple infections, as different HPV types that are amplified from one sample correspond to multiple fluorescence peak patterns in GENESCAN analysis. In this case, we performed type-specific PCR for the common HPV types 6, 11, 16, 18, 31, 33 and 51. When a positive type-specific result was obtained, we used that information to resolve ambiguous sequences. We found these common types involved in all samples that harboured more than one type. The procedure described was therefore used successfully to type multiple infections.

Of the 948 PCR-positive samples, 648 were typed. In 14% (91/648) of HPV-positive samples, more than one HPV type was present. We found 88 patients with double infections and three with triple infections. Thus, the total number of HPV isolates was 742. In all positive samples that were subjected to sequence analysis, HPV-specific sequences were obtained, demonstrating the high specificity of the assay. Typing of HPVs resulted in 39 different types. Frequencies of individual HPV types are presented in Fig. 1. The most frequently found HPV type, 16, was found at a frequency of 26.2%, followed by HPV 31 and 18 at 10.1 and 5.3%, respectively. These three types account for 41.6% of sequences. This group is followed by HPV types 33, 42, 51, 53, 58, 61, 66 and 70, which all

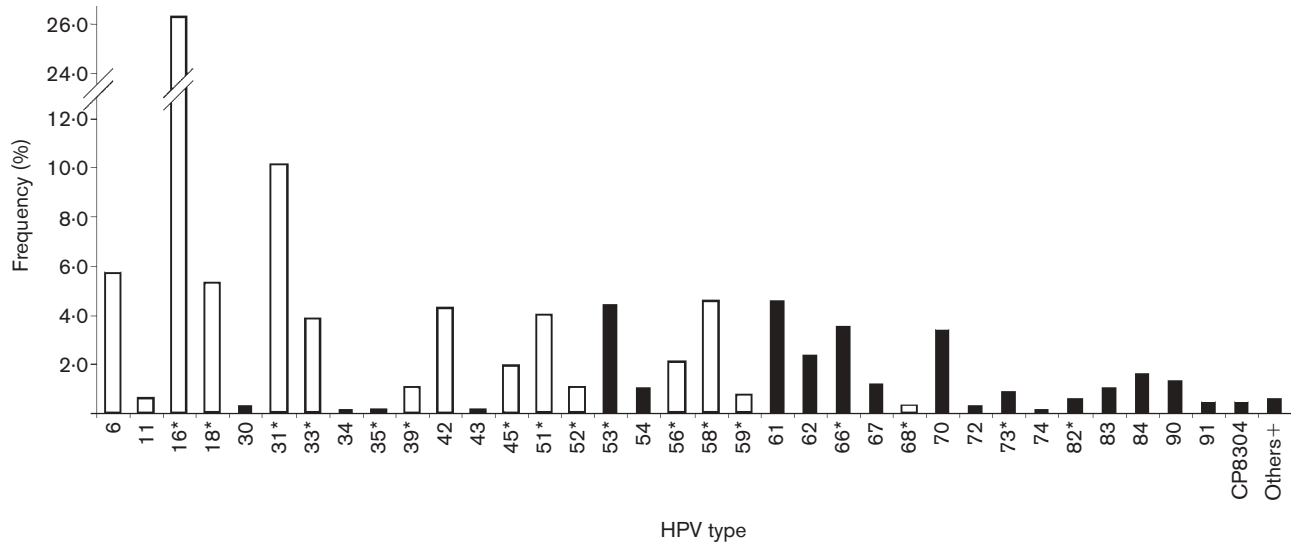


Fig. 1. Distribution of 742 HPV sequences detected by PCR-based sequencing in 648 samples. Open bars, HPV types included in the HCII test; *, HPV types classified as high-risk or probably high-risk, according to Muñoz *et al.* (2003); +, HPV types CP6108, JC9710, IA09 and mM7.

appear at a medium frequency of 3.4–4.4 %. All other types were detected at frequencies < 2.5 % and eight HPV types were detected only once.

In order to confirm our typing results, 50 samples that tested positive by our method were also analysed with the INNO-LiPA line probe assay. The results obtained were mostly concordant with our analysis. Of the samples tested, only two were negative by the INNO-LiPA assay. These samples contained HPV genotypes (61 or 84) that were not included in the type-specific probes of the INNO-LiPA assay.

Results of the HPV tests were in good agreement with cytological diagnosis of the samples. Women with HSIL all showed infection with high-risk HPV types, which were mostly HPV 16, 18, 31, 33 or 66. When a positive HPV test was obtained in cases of non-atypical cytology, the corresponding cytological sample was re-evaluated by a pathologist. All samples of this group showed either classic (koilocytosis) or non-classic cytopathological effects of HPV infection, such as mild dyskeratosis, bi- or multinucleation and nuclear hyperchromatism.

Type-specific HPV tests that are based on PCR and DNA sequencing are helpful and reliable tools in cervical cancer screening and diagnosis from cytological material, as well as from biopsies (Jacobs *et al.*, 1999; Feoli-Fonseca *et al.*, 2001; Kösel *et al.*, 2003). These findings are also confirmed by our study. All currently known high- and low-risk genital HPV types were found in our samples, with rates that are similar to published data (Bosch *et al.*, 1995; Feoli-Fonseca *et al.*, 2001; van Doorn *et al.*, 2002; Kösel *et al.*, 2003). In a recent study of a German population (Kösel *et al.*, 2003), only 5 % mixed infections were detected. In contrast, we found more than

one HPV type in 14.1 % of HPV-positive samples. Other studies report mixed infections in up to 22 % of HPV-positive samples (van den Brule *et al.*, 2002).

In our study, a significant rate of uncommon types was detected. The rates of these types are similar to the results of Kösel *et al.* (2003). Especially, HPV types such as 34, 53, 66, 73, 82 and 83 are important for early detection of cancer, as they are classified as high-risk, probably high-risk or unknown risk (Meyer *et al.*, 2001; Muñoz *et al.*, 2003) and are not included in the HCII assay. In contrast to other studies (Feoli-Fonseca *et al.*, 2001; Kösel *et al.*, 2003), a relatively high frequency of HPV 61 genotypes (4.5 %) was also observed in our population. This type is classified as low-risk by Muñoz *et al.* (2003). In contrast, we found an association of this type with aneuploidy in cases of SIL in a previous study (Bollmann *et al.*, 2003a).

Our study shows a complex distribution of HPV types in the population of the west German area (North Rhine–Westphalia). Hybridization assays are therefore limited in detecting HPV infections. Many of the sequences found in our samples (28 %) were not included in the hybrid capture probe mixture. HPV-typing systems that use PCR and DNA sequencing are useful to provide clinicians and pathologists with relevant information about HPV infection in their patients. In about 16 % of CIN that has been treated (e.g. by conization), the precancerous lesion recurs (Nobbenhuis *et al.*, 2001). Follow-up is therefore needed for risk assessment: HPV negativity 6 months after treatment has a negative predictive value of 99 % that CIN will not recur. High-risk HPV positivity has a higher sensitivity (90 %) to detect recurrent CIN than cytology alone (62 %), with equal specificity (Nobbenhuis *et al.*, 2001). Persistence of HPV

infection bears a high risk for recurrence of CIN or cancer, but only type-specific analysis can differentiate between true persistence of a specific type or a new HPV infection. This is especially important in cases of multiple HPV types (14 % in our material). HPV sequencing studies in various populations will also improve knowledge of the epidemiological distribution of HPV types, as a necessary basis for vaccine development that is targeted at uncommon genotypes.

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