Detection and typing of human papillomaviruses present in fixed and stained archival cervical smears by a consensus polymerase chain reaction and direct sequence analysis allow the identification of a broad spectrum of human papillomavirus types

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Human papillomaviruses (HPVs) are small circular DNA viruses, the >65 types of which (de Villiers, 1989) can be divided into two groups based on their tissue tropism: members of one group infect predominantly mucosal epithelia and members of the other are preferentially present in cutaneous epithelia. Approximately 25 HPV types have been detected in the anogenital tract, and epidemiological and biological evidence has pointed to a role for some of these viruses in the pathogenesis of cervical cancer (Munoz, 1988; zur Hausen, 1991).

For epidemiological purposes, methods based on the polymerase chain reaction (PCR) (Saiki et al., 1988) have been designed for the detection and typing of HPV DNA in fresh or frozen cervical biopsies (Li et al., 1988; Manos et al., 1989), unprocessed cervical smears (van den Brule et al., 1990), paraffin-embedded tissue specimens (Shibata et al., 1988; Cornelissen et al., 1989) and archival cervical smears (Rakoczy et al., 1990). The prevalence of HPV in cervical smears from women participating in a screening programme for cervical cancer (van den Brule et al., 1991) and specific groups of patients (Young et al., 1989; Manos et al., 1990; van den Brule et al., 1990, 1991) has been reported.

Determination and typing of HPV in fixed and stained archival cervical smears could be of use in assessing the prevalence of HPV types in the general population or in specific cohorts, and at different times when smears have been stored. Recently, the prevalence of HPV infection has been shown to decrease with age (Ley et al., 1991), suggesting that some HPV infections are transient or that the prevalence of HPV is increasing. Determination of its prevalence in well-defined archival Papanicolaou (Pap) smears could be used for the evaluation of these two alternatives. Furthermore, such a method could be of particular use in retrospective longitudinal studies aimed at determining the value of HPV DNA detection for the prediction of cervical cancer. Moreover, as the detection of HPV in cytological smears generally does not interfere with routine diagnostic practice, this could be of use in studies aimed at determining the effects of medical interference (e.g. biopsies, laser therapy or cryotherapy) on the persistence of HPV infection.

For the PCR amplification of a broad spectrum of HPV types, we selected a primer set (set A) consisting of two degenerate primers located in the 5' portion of the conserved E1 open reading frame (ORF). The primers were designed using a sequence similarity matrix analysis (Higgins & Sharp, 1988) of all sequenced genital HPV types, including HPV-6 (Schwarz et al., 1983), -11 (Dartmann et al., 1986), -16 (Seedorf et al., 1985), -18 (Cole & Danos, 1987), -31 (Goldsborough et al., 1989), -33 (Cole & Streeck, 1986), -39 (Volpers & Streeck, 1991) and -51 (Lungu et al., 1991). The nucleotide sequence
of the selected 5' primer was 5' ATGTTAAT(A/T)(G/C)AGCC(A/T)CCAAAATT and that of the 3' primer 5' TTATCA(T/A)ATGCCCA(T/C)TGTTACCAC. The oligonucleotides were synthesized on an Applied Biosystems 381A DNA synthesizer and purified by thin-layer chromatography before use. PCR amplification was performed according to the method of Saiki et al. (1988) in 0.1 ml of reaction mixture containing 50 mM-KCl, 10 mM-Tris–HCl pH 8.8, 3.6 mM-MgCl2, 0.01 mg/ml BSA, 0.2 mM of each dNTP, 2 units of Taq DNA polymerase (Perkin-Elmer Cetus) and 300 ng of each primer. Forty cycles of amplification (1 min at 95 °C, 1 min at 55 °C and 2 min at 72 °C) were performed in a Perkin-Elmer Cetus thermal cycler. The predicted 188 bp fragment of the E10RF (nucleotides 1777 to 1964 in the case of HPV-16) was amplified from nine of 10 genital (Fig. 1) and 20 of 22 skin HPV types tested using this primer set. Of the skin HPV types, types 1, 2, 3, 4, 5, 7, 8, 10, 14, 19, 20, 21, 22, 23, 24, 25, 36, 37, 46 and 49 were amplified, whereas types 41 and 50 were not (data not shown).

To permit typing by sequence analysis, PCR products were reamplified using the same primer set and sequenced using the 3' primer of set A. The PCR product was extracted once with phenol/chloroform/isoamyl alcohol (25:24:1), precipitated with ethanol, dried, dissolved in 15 μl TE buffer and run on a 2% agarose gel. The ethidium bromide-stained 188 bp HPV fragment was excised from the gel and the DNA recovered from the agarose using the guanidinium isothiocyanate (GTC)–silica procedure (Boom et al., 1990). Sequence analysis was done directly on the DNA recovered, using Sequenase version 2.0 and the USB sequencing kit according to the manufacturer's protocol, except that denaturation of the DNA and subsequent annealing of the primer were performed by boiling for 5 min and fast cooling at -70 °C. Generally the sequences could be read for about 100 nucleotides, and gave sufficient information to distinguish between all amplified genital and skin HPV types (data not shown). Therefore consensus primer set A can be used to detect and type a wide spectrum of known and presumably also unidentified genital and skin HPV types.

To determine whether HPV detection and typing can be done on nucleic acid extracted from fixed and stained archival Pap smears, 62 randomly selected cervical smears stored for between 11 and 13 years were selected. The smears had been taken from women attending the outpatient clinic of the Academic Medical Centre for a number of reasons, and had been prepared for routine cytological examination by fixation in 96% ethanol, staining and embedding in malinol. According to the cytological diagnosis, which was made essentially following the criteria described by Ferenczy (1982), as well as smears classified as Pap I or Pap II, smears containing dysplastic (Pap III and IV) or cancerous (Pap V) cells were included (Table 1). For extraction of nucleic acid, slides were placed in xylene in individual containers with the coverslip positioned upwards, and left for 40 h. The coverslips were removed, and cell material was collected and transferred into an Eppendorf vial using a fresh razor blade. Subsequently, nucleic acid extraction was performed according to the GTC–silica procedure. Briefly, 0.9 ml cell lysis buffer (prepared by dissolving 120 g GTC and 2.6 g Triton X-100 in 100 ml 0.1 M-Tris–HCl pH 6.4, 0.2 ml 0.2 M-EDTA pH 8-0) was added to the cell material and mixed vigorously. After incubation for 30 min at room temperature, 0.04 ml of a sterile suspension of activated silica (coarse) in 0.1 M-HCl was added and the mixture was incubated for an additional 10 min with occasional shaking. The silica and bound nucleic acids were collected by a 30 s centrifugation step, the pellet was washed twice with 1 ml wash buffer (prepared by dissolving 120 g GTC in 100 ml 0.1 M-Tris–HCl pH 6.4) by thorough mixing and centrifugation and subsequently washed once with 70% ethanol and acetone. Finally, the silica was dried by incubation at 58 °C for 15 min and the nucleic acids were eluted from

<table>
<thead>
<tr>
<th>Class</th>
<th>No. of cases</th>
<th>HPV-positive HPV type (no. of each type)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pap I</td>
<td>6</td>
<td>1 HPV (1)</td>
</tr>
<tr>
<td>Pap II</td>
<td>38</td>
<td>13 HPV (7); 33 (1); 51 (1); X* (4)</td>
</tr>
<tr>
<td>Pap IIIa</td>
<td>9</td>
<td>6 HPV (1); 31 (1); 33 (1); 45 (1); 51 (1); X (1)</td>
</tr>
<tr>
<td>Pap IIIb</td>
<td>1</td>
<td>1 HPV (1)</td>
</tr>
<tr>
<td>Pap IV</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Pap V</td>
<td>5</td>
<td>5 HPV (2); 31 (2); 45 (1)</td>
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* X, Unidentified type.
The virus type in all HPV-positive Pap smears was determined by sequence analysis of the 188 bp HPV DNA fragment purified after amplification by two consecutive PCRs of 40 cycles each. In Fig. 3, the sequences of the 188 bp fragment of seven HPV types detected in smears, including those of the HPV-positive smears shown in Fig. 2, are presented. As summarized in Table 1, all five smears suspected to contain cancerous cells (Pap V) contained an oncogenic HPV type; the two smears classified as Pap V and containing HPV-31 were taken from the same patient. In the 10 HPV-positive smears classified as Pap III, six contained an oncogenic HPV type and one an unidentified type. In the smears classified as Pap I and II, known oncogenic as well as unidentified HPV types were detected. In 22 cases, the HPV DNA was identified by comparison with reference sequences: HPV-1 (Danos et al., 1982), -2 (Hirsch-Beahn et al., 1990), -3, -4, -5 (Zachow et al., 1987), -6 (Schwarz et al., 1983), -8 (Fuchs et al., 1986), -10, -11 (Dartmann et al., 1986), -12, -14, -15, -16 (Seedorf et al., 1985), -17, -18 (Cole & Danos, 1987), -20, -21, -24, -25, -31 (Goldsborough et al., 1989), -33 (Cole & Streeck, 1986), -35, -36, -37, -38, -39 (Voipers & Streeck, 1991), -41 (Hirt et al., 1990), -42 (Phillip et al., 1992), -45, -46, -47 (Kiyono et al., 1990), -49, -51 (Lungu et al., 1991), -57 (Hirsch-Beahn et al., 1990) and -58 (Kiri et al., 1991). The sequences of the HPV types for which no sequence information has been published were obtained by sequence analysis of the 188 bp PCR amplification product. The unidentified HPV types detected in the smears showed less than 85% identity with each of the reference HPV types, although two were identical.

The prevalence of HPV DNA in cervical scrapes depends on the method of selection as well as the composition of the study group, and the number of HPV-positive smears increases with the severity of the lesion according to the Pap classification (van den Brule et al., 1991; Bauer et al., 1991). Van den Brule et al. (1991) have reported prevalences of 70%, 84% and 100% respectively in smears with mild and severe dysplastic abnormalities and smears suspected of showing carcinoma in situ. The present study group was composed of women attending a gynaecological outpatient clinic for various reasons. Given the relatively high proportion of smears containing dysplastic cells in the archival smears analysed in this study, the 40% HPV positivity is expectedly high.

The detection of HPV-16, -31 and -45 DNA in smears suspected of harbouring cancerous cells (Pap V), and the detection of HPV-16, -18, -31, -35, -45 and -51 DNA in extracted from the archival Pap smears, it appeared to be essential that the selected PCR primer set amplified a DNA fragment of limited length; a primer set amplifying a 260 bp fragment of the human globin gene gave negative results in most cases.

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smears containing dysplastic cells (Pap IIIa and IIIb) is in agreement with the oncogenic character of these viruses (Woodworth et al., 1989; zur Hausen, 1991) and with the presence of these and some other HPV types in cervical carcinomas and high grade cervical intraepithelial neoplastic lesions (Meanwell et al., 1987; Wilczynski et al., 1988; Resnick et al., 1990; Riou et al., 1990; van den Brule et al., 1991; Yoshikawa et al., 1991; Tham et al., 1991; Cornelissen et al., 1992). However, a small percentage of cervical carcinomas contain as yet unidentified HPV types and additional studies are required to identify the whole spectrum of types present in cervical carcinomas. Consensus primer pair A could be useful for the detection of such types.

Screening programmes to detect women with premalignant or malignant cervical lesions are generally based on cytology. The strong correlation between the detection of HPV DNA and the presence of dysplastic
cells and koilocytes in histological sections of the cervical epithelium (Cornelissen et al., 1989; Syrjanen & Syrjanen, 1988) argues strongly for the presence of such cells in the cervix of women from whom these HPV-positive smears had been taken, even if the smears were negative according to cytological criteria. The described procedure to detect HPV DNA in fixed and stained archival cervical smears could be of particular use in assessing the influence of HPV infection on the clinical outcome. Moreover, the method could be useful in retrospective epidemiological studies for assessing the reliability of HPV detection and typing compared with cytological screening methods in the prediction of women at risk of the development of cancer because prospective studies cannot be performed owing to ethical reasons.

The HPV typing procedure involving direct sequence analysis of a PCR product may be particularly useful in those instances where a large number of HPV types must be discriminated because it overcomes the requirement for a large number of type-specific probes and substitutes laborious multiple hybridization steps for a single analysis procedure. The workload could potentially be reduced further by the incorporation of a non-radioactive sequence analysis method, sequencing using ssDNA PCR amplification and automatic sequencing equipment.

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References


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