Rapid real time PCR to distinguish between high risk human papillomavirus types 16 and 18

H A Cubie, A L Seagar, E McGoogan, J Whitehead, A Brass, M J Arends, M W Whitley

Abstract

Aims—To assess the validity and practicality of real time polymerase chain reaction (PCR) for human papillomavirus (HPV) testing in combination with liquid based cytology samples for cervical screening.

Methods—Real time PCR using consensus (GP5+/6+) and type specific primers was developed to detect genital HPV types. This provides rapid, efficient amplification followed by denaturation of the product and computer analysis of the kinetics data that are generated. Liquid based cytology samples were obtained from patients attending routine cervical screening clinics. DNA was extracted from the residual cellular suspension after cytology using spin columns.

Results—Real time PCR successfully distinguished between HPV-16 and HPV-18 on the basis of amplification with consensus primers followed by DNA melting temperature (Tm) analysis. Sensitivities of one to 10 copies of HPV-16 (mean Tm = 79.4°C; 2 SD, 0.8) and four to 40 copies of HPV-18 (mean Tm = 80.4°C; 2 SD, 0.4) were obtained. In a mixed population of SiHa and HeLa cells containing known copy numbers of HPV-16 and HPV-18 genomes, HPV-16 and HPV-18 products were clearly separated by Tm analysis in mixtures varying from equivalence to 1/1000. Together with detailed melt analysis, type specific primers from the same region of the L1 gene confirmed the differential ability of this system. The method was applied to 100 liquid based cytology samples where HPV status using conventional GP5+/6+ PCR was already known. There was 95% agreement between the methods, with 55 positives detected by conventional PCR and 59 with real time PCR. The method was then tested on 200 routine liquid based cytology samples. Approximately 10% were positive by real time PCR, most of which were classified as HPV-16 by detailed melt analysis. Thirteen (6.8%) HPV positives were identified in 189 samples showing no evidence of cervical cytological abnormality.

Conclusions—Real time PCR is a rapid, efficient method for the detection of HPV with the separation of HPV-16 and HPV-18 on the basis of differential Tm.

Keywords: real time polymerase chain reaction; cervical screening; human papillomavirus types 16 and 18

More than 40 types of human papillomavirus (HPV) infect the genital epithelium and several high risk types including HPV types 16, 18, 31, 33, and 45 are found in almost all cases of high grade cervical intraepithelial neoplasia and cervical cancer.1,2 In Europe, the most prevalent type is HPV-16,1 but there are several reports that HPV-18 infection can lead to the development of more clinically aggressive disease.3,4 Laboratory diagnosis of HPV infection is dependent upon molecular techniques such as DNA hybridisation or nucleic acid amplification. Several polymerase chain reaction (PCR) methods have been developed to detect a broad spectrum of mucosotropic HPV types using either degenerate or consensus primers.5,6 A second generation commercial hybridisation assay, Hybrid Capture™ (HCA II), is also available for the detection of HPV DNA in cervical swab samples,10 and has been used widely in epidemiological studies.11–13 However, both consensus PCR and HCA II have important limitations. They are costly and labour intensive and, without additional procedures, neither technique can differentiate between individual types or detect infection with more than one type. Furthermore, HPV infections are often transient, frequently cleared by immunocompetent people, and require interaction with cofactors for the progression of disease. Thus, the development of highly sensitive detection tests for high risk HPV raises problems of clinical interpretation.

The potential use of HPV testing in cervical screening programmes is dependent on a rapid sensitive test that can distinguish high risk HPV types present in clinical samples. In most conventional PCR assays, amplification is performed by automated temperature cycling, but product analysis requires a subsequent manual operation.

Rapid real time PCR can distinguish closely related sequences on the basis of amplification followed by DNA melting temperature analysis. The commercial LightCycler (Idaho Technology Inc, supplied by BioGene Ltd, Kimbolton, Cambridgeshire, UK) combines simultaneous PCR amplification with sophisticated computer analysis of the kinetics data generated. The use of air as a circulating medium during PCR cycling allows rapid temperature control and thus a significant reduction in testing time (typically 40 cycles in 25 minutes). The use of fine capillaries of borosilicate glass provides efficient heat transfer and by acting as wave guides facilitates sen-
sitive fluorimetry and enhances the efficiency of the amplification. The amplification mix contains a fluorescent dye, SYBR Green I<sup>13</sup>, which binds to the minor groove of double stranded DNA and emits light on excitation.<sup>14</sup> Thus, as the PCR product accumulates, fluorescence increases. On denaturation of the product, SYBR Green I is released and fluorescence rapidly decreases. Because the melting curve of DNA is dependent on sequence, length, and GC content, PCR products can be distinguished by their melting curves. The determination of melting curves can be carried out on each sample after amplification without opening the reaction vessels. We report the differentiation of HPV-16 and HPV-18 in mixed cell populations using GP5+/6+ consensus primers,<sup>9</sup> with confirmation without opening the reaction vessels. The determination of melting curves can be distinguished by their melting curve: DNA is dependent on temperature, base variations between types, and the possibility of non-specific amplification. The amplification mix contains 0.5 µl of 5 µM forward and reverse primers, 1.25 µl of template nucleic acid, 0.25 µl of 1/1000 SYBR Green I, 4 mM MgCl<sub>2</sub> (Biogene Ltd) and TaqStart enzyme (BamHI, EcoRI, HindIII, HindII, and BglII) and electrophoresed in 1% agarose to check the identity of the DNA. Finally, GeneQuant II spectrophotometric analysis (Amersham Pharmacia Biotech, St Albans, Hertfordshire, UK) was performed to measure the concentration of DNA.

**CLINICAL SAMPLES**

A total of 300 liquid based cytology samples were collected from women attending general practitioner clinics for routine cervical screening or follow up. Cells were collected from the cervix using a Cervex Brush® rinsed in 20 ml of PreservCyt® (PC) solution (Cytyc Corporation; Boxborough, Massachusetts, USA). The sampler was then discarded. One hundred specimens were “split samples”, where a conventional smear had been made before the residual cervical material was rinsed into the liquid based cytology medium. The other 200 samples were collected routinely and only a ThinPrep® (TP) monolayer smear was made for diagnostic purposes.

**DNA EXTRACTION**

Cells from the residual volume were pelleted at 2900 ×g for 15 minutes before resuspending in 200 µl of Tris EDTA (TE) buffer (pH 7.2). DNA extraction was then performed directly using the Qiagen DNA mini kit, according to the manufacturer's tissue protocol, resulting in 400 µl of extracted sample.

**PRIMERS AND PCR PROTOCOL**

Three primer pairs were used in our study: the GP5+/6+ consensus primer pair, together with HPV-16 and HPV-18 specific primer pairs, which were modified from GP5+/6+ (table 1). These were designed after a thorough search of the HPV sequence database (Los Alamos National Laboratory) in the GP5+/6+ primer target region of the L1 gene. The DNA sequence of all known HPV types was critically reviewed for optimal product length, annealing temperature, base variations between types, and the possibility of non-specific amplification.

Reaction mixes contained 0.5 µl of 5 µM forward and reverse primers, 1.25 µl of template nucleic acid, 0.25 µl of 1/1000 SYBR Green I, and 2.5 µl of master mix containing 4 mM MgCl<sub>2</sub> (Biogene Ltd) and TaqStart antibody (Sigma-Aldrich Co Ltd, Poole, Dorset, UK). The cycling profiles were optimised for each set of primers and are detailed in table 1.
Table 2  Detailed melt analysis of DNA extracted from SiHa and HeLa cells

<table>
<thead>
<tr>
<th></th>
<th>Number of replicates</th>
<th>(T_m) with GP5+/6+ (mean (T_m) (2 SD))</th>
<th>Number of replicates</th>
<th>(T_m) with TS primers (mean (T_m) (2 SD))</th>
</tr>
</thead>
<tbody>
<tr>
<td>SiHa</td>
<td>20</td>
<td>79.1 (0.8°C)</td>
<td>30</td>
<td>78.2 (0.4°C)</td>
</tr>
<tr>
<td>HeLa</td>
<td>20</td>
<td>80.9 (1.0°C)</td>
<td>30</td>
<td>80.4 (0.3°C)</td>
</tr>
</tbody>
</table>

1. Detailed melt analysis was used for the accurate determination of the melting point of the amplified product. This consisted of a single cycle of 65°C for three seconds to 90°C for one second at a transition rate of 0.2°C/second, followed by measurement of the fluorescent signal at greater frequency. The entire assay including DNA extraction, PCR amplification, and melting temperature analysis can be performed in approximately three hours.

HYBRID CAPTURE ASSAY
A 4 ml volume of PC fluid was processed for the Digene HPV hybrid capture assay (HCA) according to the manufacturer's recommendations and using the second generation (HCA II) test. This is a sandwich capture hybridisation system using chemiluminescent signal amplification for the qualitative detection of 13 different high risk HPV types (HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68). The emission of light is measured as relative light units (RLUs) and is proportional to the amount of target DNA present. Samples with an RLU > 1 were considered positive for any of the high risk HPV types contained in the probe pool. HPV results were correlated with the cytology results obtained.

CYTOLOGICAL ASSESSMENT
TP slides were made using the semi-automated ThinPrep 2000 slide processor (Cytyc Corporation, Boxborough, Massachusetts, USA). The methodology has been well documented elsewhere. Both conventional and TP smears were reported independently. The Richart system for cervical diagnostic reporting was used (unsatisfactory (U/S), negative (WNL), borderline changes (B/L), mild dyskaryosis, moderate dyskaryosis, and severe dyskaryosis).

Figure 1  Melting curve analysis of PCR products after amplification of a 150 bp fragment of the human papillomavirus (HPV) L1 gene. Y axis: negative differential of fluorescence over temperature (−dF/dT). (A) Standard melt analysis of SiHa/HeLa mixed DNA product, using GP5+/6+ primers showing single peak (\(T_m\) = 79.4°C). (B) Detailed melt analysis of SiHa and HeLa mixed DNA product, showing two peaks with \(T_m\) = 78.4°C (HPV-16) and 80.1°C (HPV-18).
Table 3  Detection of human papillomavirus 16 (HPV-16) in a background of HPV-18 with GP5+/6+ and with TS 165+/166+ primers

<table>
<thead>
<tr>
<th>HPV type</th>
<th>Standard melt Tm</th>
<th>Detailed melt Tm</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV-33</td>
<td>76.7°C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPV-31</td>
<td>78.0°C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPV-45</td>
<td>78.2°C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPV-16</td>
<td>79.4°C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPV-18</td>
<td>80.4°C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPV-45</td>
<td>78.2°C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPV-16</td>
<td>79.4°C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPV-18</td>
<td>80.4°C</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4  Melting temperature (T_m) of high risk human papillomavirus (HPV) cloned material using real time PCR with GP5+/6+ primers and detailed melt analysis

<table>
<thead>
<tr>
<th>HPV type</th>
<th>T_m</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV-33</td>
<td>76.7°C</td>
</tr>
<tr>
<td>HPV-31</td>
<td>78.0°C</td>
</tr>
<tr>
<td>HPV-45</td>
<td>78.2°C</td>
</tr>
<tr>
<td>HPV-16</td>
<td>79.4°C</td>
</tr>
<tr>
<td>HPV-18</td>
<td>80.4°C</td>
</tr>
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</table>

Table 5  Comparison of conventional and real time PCR on 100 selected clinical samples

<table>
<thead>
<tr>
<th>HPV type</th>
<th>Conventional PCR results</th>
<th>Light Cycler PCR results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Equivocal</td>
</tr>
<tr>
<td>HPV-18</td>
<td>55</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 6  Human papillomavirus (HPV) findings related to cytology in 200 unselected, routine liquid based cytology samples

<table>
<thead>
<tr>
<th>Cytology</th>
<th>Total number of samples</th>
<th>HPV positive</th>
<th>HPV negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unsatisfactory</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Borderline</td>
<td>189</td>
<td>13 (6.8%)</td>
<td>176</td>
</tr>
<tr>
<td>Mild dyskaryosis</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Moderate dyskaryosis</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Severe dyskaryosis</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>200</td>
<td>19 (9.5%)</td>
<td>181</td>
</tr>
</tbody>
</table>

Discussion

We have shown that HPV-16 and HPV-18 can be detected by rapid real time PCR using consensus primers and can be differentiated by melting curve analysis. In mixed samples, two separate peaks of distinct T_m are seen, even when there is a considerable difference in the copy number of each type. This approach has been confirmed with type specific primers.

Discussion

We have shown that HPV-16 and HPV-18 can be detected by rapid real time PCR using consensus primers and can be differentiated by melting curve analysis. In mixed samples, two separate peaks of distinct T_m are seen, even when there is a considerable difference in the copy number of each type. This approach has been confirmed with type specific primers.

Because HPV-16 and HPV-18 product lengths are very similar, it is impossible to differentiate between individual HPV types by agarose gel electrophoresis after GP5+/6+ PCR amplification. In contrast, melting curve analysis using LightCycler technology can distinguish between products of the same length but different GC : AT ratios. Woo and colleagues reported the use of genus specific amplification primers and specific fluorogenic hybridisation probes to differentiate pathogenic and non-pathogenic strains of leptospira. In their study, the lower limit of detection was 200 genome copies. The same group also used melting curve analysis to differentiate different strains of leptospira and to distinguish reference strains and field isolates of leptospira without the use of hybridisation probes. More recently, reports of the use of the LightCycler in detecting viruses have begun to appear. These include the quantitative detection of human cytomegalovirus (CMV) in plasma and the sensitive diagnosis of herpes simplex virus in clinical samples using T_m analysis to distinguish between herpes simplex virus 1 (HSV-1) and HSV-2. We have used a similar approach to separate HPV types, specifically for the differentiation of HPV-16 and HPV-18 products that differ in T_m by less than 2°C.

Real time PCR technology has great potential for clinical and non-clinical development. Nevertheless, it is still a new technique and some technical problems have been reported, including the presence of primer dimer formation. In our hands this could be minimised by careful attention to optimisation conditions, with very small changes in concentration, temperature, and the use of TaqStart antibody having a considerable effect on the shape of the analytical trace. In addition, the presence of a high molecular weight non-specific PCR product has been noted in some applications.
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Figure 2 Melting curve analysis of PCR products from clinical liquid based cytology samples. (A) Detection of a single peak of human papillomavirus 16 (HPV-16) using detailed melt analysis. (B) Detection of a mixed infection with HPV types 16 and 18 using detailed melt analysis.

twer and colleagues suggested that “shoulders” of non-specific PCR product might be caused by substantial product to product annealing in later amplification cycles. A small shoulder was sometimes seen in our study on the high temperature side of the melting curve. The problem was minimised by reducing the amplification mix from 10 µl to 5 µl and indeed the manufacturers recommend a volume of 5–7 µl as optimal in each capillary. Larger volumes can result in uneven temperature distribution and therefore inefficient amplification towards the top portion of the capillary. With optimal conditions and volume, we only observed this phenomenon occasionally with type specific primers and it did not interfere with the determination of the Tm value. The effect was never seen with the consensus primers. Quality control of the reagents used is undoubtedly important to limit variations in Tm and positive control material was included and fully analysed in every run.

Additional practical problems have included the fragility of the capillaries. However, breakages were minimal with experienced operators and the second generation LightCycler produced by Roche Molecular Systems uses more robust capillaries. The extended use of the LightCycler for detailed melt analysis can result in overheating of the carousel and, in our hands, only 10 detailed melt analyses could be carried out before a 30 minute cooling period was required. Nevertheless, the rapid cycling time with standard melts allowed up to seven PCR runs to be carried out in a single working day.

We used melting point analysis after amplification in a single reaction for the detection of single and mixed samples of HPV-16 and HPV-18 in both cell lines in vitro and in cervical secretions from patients, including 100 samples validated by both conventional PCR and HCA II. Subsequently, the protocol was applied successfully to 200 liquid based cytology clinical samples, with HPV DNA being detected in 9.8% of samples (table 6), including 13 of 189 (6.8%) showing no cytological abnormality. Although this is consistent with other studies, analysis of the HPV results in relation to cervical dyskaryosis requires a much larger study group and this work is currently under way. The combined approach of rapid amplification and product identification in a single PCR reaction is an exciting one, with great potential for both clinical and non-clinical development, particularly in terms of introducing HPV testing into cervical screening programmes. Opportunities for high throughput are possible by combining one of several available robotic handling instruments for DNA extraction with the LightCycler system and we are testing the maximum daily capacity of such combinations.

We are grateful to Professor E-M de Villiers (Referenzzentrum fur humanpathogene Papillomviren, Heidelberg, Germany) for providing HPV types 16, 18, and 45; Dr G Orth (Institut Pasteur, Paris, France) for HPV-33; and Dr A Lorincz (Digene Diagnostics, Silver Spring, Maryland, USA) for HPV-31. We acknowledge the help of Dr B Morris, department of pathology, University of Edinburgh with growth and isolation of the plasmid DNAs. We would like to thank the Chief Scientist Office of the Scottish Executive for funding this work (Grant No K/MRS/50/C2699) and the NHS R&D Support Fund for additional support.


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