

# Amplification with molecular beacon primers and reverse line blotting for the detection and typing of human papillomaviruses

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## Abstract

A novel method for the detection and typing of human papillomavirus (HPV) was developed using molecular beacon primers. The method is based on the use of HPV-specific primers containing a hairpin loop structure in which fluorescent donor and quencher groups are held in close proximity such that fluorescence is quenched. Amplification of the target sequence results in the opening of the loop and the resulting fluorescence can be detected on a sequence detector system (SDS) 7700 (Applied Biosystems), as used for TaqMan™ assays. Fluorescent amplicons were identified on the SDS 7700 and then typed by a single hybridisation with specific probes immobilised in lines on a nylon membrane and detected on a fluorescent scanner. This novel beacon primer method compared well with conventional PCR for cervical scrape specimens. The combination of the beacon primer method and reverse line blotting should enable large-scale population studies of HPV infection. © 2000 Elsevier Science B.V. All rights reserved.

*Keywords:* Human papillomavirus; Line blotting; Molecular beacon primer; Sequence detector system

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## 1. Introduction

Human papillomaviruses (HPVs) are classified into approximately 100 genotypes, defined on the

basis of DNA sequence of the L1 (capsid), E6, and upstream regulatory regions (van Ranst et al., 1993). Sexually transmitted HPV infections in women divide into symptomatic warts on the external genitalia, most commonly caused by types 6 or 11, and asymptomatic cervical infections. A small proportion of women with cervical HPV infection progress to cervical intraepithelial

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neoplasia (CIN) which, if not detected and treated, may develop into cervical cancer. The risk of cervical cancer developing is the highest for HPV types 16, 18, 31 and 45 and lowest for types 6 and 11. There is growing evidence that HPV infection is an essential prerequisite for the development of cervical cancer (Walboomers et al., 1999).

Cervical cancer is the commonest cancer in women aged under 35 and is a major cause of death in this age group in developing countries such as India and Brazil which cannot resource a comprehensive cervical screening programme (Nandakumar et al., 1995). Even in Western countries an unacceptable proportion of cervical smears are falsely reported as normal (Fricker, 1997). The publicity surrounding the development of cancer in women missed by the cervical screening programme has led to the suggestion that testing for potentially oncogenic HPV viruses should be added (Cuzick et al., 1995). One suitable commercially available test (Digene Corp. Beltsville, MD) uses the technique of direct hybridisation of HPV DNA with a cocktail of probes designed to detect the most common high and intermediate risk HPV types. The limitations of such a test for studies on the natural history of cervical HPV infection in different populations include; (1) an inability to identify the infecting HPV type or even discriminate between high and intermediate risk types; (2) inability to identify mixed HPV infections; and (3) all the infections with the many HPV types not included in the probe cocktail will be recorded as negative. A significant advance was made by Gravitt et al. (1998) who used biotinylated primers for the L1 gene and probed the PCR amplification products with a panel of 27 HPV genotypes. This approach identifies the HPV types involved, including mixed infections, but records as negative HPV infections not included in the probe panel. Further, 75% of samples tested by probing were negative generating a considerable unnecessary workload.

Using molecular beacon labelled primers (Nazarenko et al., 1997) we have developed a PCR method which permits the real-time detection of any HPV infection. Further, the fluores-

cent amplicons detected in positive samples are suitably labelled for direct testing against a panel of HPV genotype-specific probes. To confirm the validity of the method for rapid screening and the detection of HPV types not covered by the probe panel, we compared it with conventional PCR in a study of cervical samples from women attending a sexually transmitted diseases (STD) clinic in Colombo, Sri Lanka.

## 2. Materials and methods

### 2.1. Cervical samples

Women attending the central STD clinic from September through December 1997 were invited to participate in the study. All the patients were interviewed in Sinhalese or Tamil and completed a questionnaire designed to identify risk factors for the acquisition of genital tract HPV infections. The 352 women who agreed to participate in the study were managed in accordance with standard clinical procedures but with the addition of a cervical cytobrush (Cellpath, UK) sample taken for HPV detection. Cytobrushes were placed in 1ml of phosphate buffered saline (PBS) containing 0.1% sodium azide as preservative and stored frozen at  $-20^{\circ}\text{C}$ . The study protocol was approved by the ethical committee of the National Hospital of Colombo.

### 2.2. Extraction of HPV DNA

Cells and HPV were detached from the sampling device by vortex mixing and the sample centrifuged at  $60\,000 \times g$  for 30 min at  $20^{\circ}\text{C}$ . DNA was extracted from the pelleted cells and HPV by alkali lysis. Briefly, 200  $\mu\text{l}$  2 M ammonium hydroxide was added to the pellet and the sealed tubes heated at  $90^{\circ}\text{C}$  for 10 min. To remove the ammonia totally, the tubes were uncapped and reheated at  $90^{\circ}\text{C}$  for 70 min, leaving a residual volume of approximately 100  $\mu\text{l}$ . The control cell lines CaSki (HPV-16) and HeLa (HPV-18) were processed in the same way.

### 2.3. PCR amplification and detection

All the samples were tested for HPV using a conventional PCR with GP5+ and GP6+ primers (de Roda Husman et al., 1995) to which 5' fluorescein had been added to the GP6+ primer to enable typing by reverse line blotting. Amplification reactions contained 1 × buffer (Promega, UK), 1.5 mM MgCl<sub>2</sub>, 250 μM each of dATP, dCTP, dGTP, and dTTP, 1 μM each primer, 1.25 U Taq polymerase (Promega, UK) and 10 μl of sample extract in a final volume of 50 μl. Amplification parameters were 40 cycles of 30 s at 94°C, 30 s at 40°C and 20 s at 72°C on a Perkin–Elmer 9600 thermal cycler. Products were detected by ethidium bromide staining of agarose gels and stored at –20°C until required for reverse line blotting.

### 2.4. Molecular beacon primer-based PCR amplification and detection

The hairpin loop of a DNA sequence shown previously to give maximum fluorescence above background when incorporated into an amplicon (Nazarenko et al., 1997) was added to the 5' end of the antisense GP6+ primer sequence. The primer sequence (ZMB2) contained a 5' fluorescein residue (F) and a quencher (Q, a dabcyil analogue on a T base) (see Fig. 1) and was synthesised by Oswel DNA services (Southampton). The optimised reaction mixtures for the molecular beacon primers contained 1 × buffer A, 3.5 mM MgCl<sub>2</sub>, 200 μM each of dATP, dCTP and dGTP and 400 μM dUTP, 1.25 U Amplitaq Gold (all provided in the Taqman™ PCR core reagent kit; PE Applied Biosystems), 300 nM GP5+ and ZMB2 primers, and 10 μl DNA extract in a total volume of 50 μl. Dilutions of HPV-16 DNA cloned in a plasmid (kindly provided by Dr J. Gray, Cambridge) containing approximately 3, 10, 10<sup>2</sup>, 10<sup>3</sup> and 10<sup>4</sup> copies/10 μl were included in duplicate in every experiment and used to construct a standard curve. At least three negative controls, containing water, were included in every experiment. Reagent mixtures were kept on ice throughout the addition of target DNA to help prevent the formation of primer dimers. Amplification conditions consisted of 10 min at 95°C to activate the polymerase followed by 60 cycles of 30 s at 94°C, 30 s at 40°C and 20 s at 72°C. Emitted fluorescence was analysed at the lowest cycling temperature (40°C), at which unincorporated beacon primers refold to their hairpin, minimal fluorescent state.

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Beacon primer sequence: 5'-F CACCTTCACCCCTCAGAAGG Q GGAAAAATAAACTGTAAATCATATTC-3'

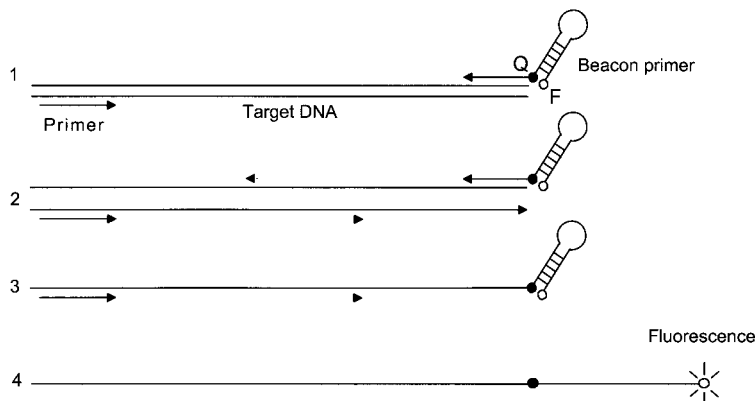


Fig. 1. Diagram showing the incorporation and opening out of molecular beacon primers during amplification, leading to fluorescence. (1), annealing of unlabelled and molecular beacon primers; (2), annealing and extension from primers; (3), annealing of unlabelled primer to molecular beacon primed DNA with extension leading to fluorescence (4), Q, quencher; F, fluorescein.

Table 1  
Probe sequences for reverse line blotting

HPV type	Probe sequence (5'–3')	References
6	ATC CGT AAC TAC ATC TTC CAC ATA CAC CAA	Jacobs et al., 1995
11	ATC TGT GTC TAA ATC TGC TAC ATA CAC TAA	Jacobs et al., 1995
16	GTC ATT ATG TGC TGC CAT ATC TAC TTC AGA	Jacobs et al., 1995
18	TGC TTC TAC ACA GTC TCC TGT ACC TGG GCA	Jacobs et al., 1995
31	TGT TTG TGC TGC AAT TGC AAA CAG TGA TAC	Jacobs et al., 1995
33	TTT ATG CAC ACA AGT AAC TAG TGA CAG TAC	Jacobs et al., 1995
35	GTC TGT GTG TTC TGC TGT GTC TTC TAG TGA	Jacobs et al., 1995
39	TCT ACC TCT ATA GAG TCT TCC ATA CCT TCT	Jacobs et al., 1995
42	CTG CAA CAT CTG GTG ATA CAT ATA CAG CTG	Jacobs et al., 1995
43	TCT ACT GAC CCT ACT GTG CCC AGT ACA TAT	Jacobs et al., 1995
45	ACA CAA AAT CCT GTG CCA AGT ACA TAT GAC	Jacobs et al., 1995
51	AGC ACT GCC ACT GCT GCG GTT TCC CCA ACA	Jacobs et al., 1995
52	TGC TGA GGT TAA AAA GGA AAG CAC ATA TAA	Jacobs et al., 1995
53	CCG CAA CCA CAC AGT CTA TGT CTA CAT ATA	This study
54	TAC AGC ATC CAC GCA GGA TAG CTT TAA TAA	This study
56	GTA CTG CTA CAG AAC AGT TAA GTA AAT ATG	This study
58	ATT ATG CAC TGA AGT AAC TAA GGA AGG TAC	This study
59	GTG CTT CTA CTA CTT CTT CTA TTC CTA ATG TAT	This study

Table 1 (Continued)

HPV type	Probe sequence (5'–3')	References
62	CTG CTG CAG CAG AAT ACA CGG C	This study
66	ATT AAT GCA GCT AAA AGC ACA TT	Hildesheim et al., 1994
67	CTG AGG GAA AAT CAG AGG CTA C	Qu et al., 1997
70	CTG CAC CGA AAC GGC CAT AC	This study
72	GCC ACA GCG TCC TCT GTA TCA GA	This study
han831	GTG CCA CAC AAA CAC CCT CTG A	This study
CP8304	CAC AGC TAC ATC TGC TGC TGC AGA	This study

### 2.5. Probe design and attachment to nylon membranes

Type-specific probes for 20 common HPV types were designed within the GP5 + /GP6 + amplification regions of the L1 gene (sense). The sequences (and references) of the probes are given in Table 1. Probes were synthesised in-house on an Expedite oligonucleotide synthesiser (Millipore), incorporating an amino-link at the 5' end. Bio-dyne nylon membranes (Pall BioSupport, UK) were activated in 16% EDAC (Sigma, UK) for 15 min, rinsed in water and placed in a miniblotted (Biometra Ltd, UK). Optimum coupling of probes to the membrane was achieved by adding 250 pmoles probe diluted in 125 µl 500 mM NaHCO<sub>3</sub> to the slots for 1 min only. The membrane was then removed from the blotter, inactivated in 100 mM NaOH for 10 min, rinsed in water and then incubated in 2 × SSPE-0.1% SDS (1 × SSPE is 180 mM NaCl, 10 mM NaH<sub>2</sub>PO<sub>4</sub>, 1 mM Na<sub>2</sub> EDTA) for 10 min at 56°C. After a final rinse in 2 × SSPE membranes were used immediately or stored at 4°C for subsequent use.

### 2.6. Hybridisation and detection by reverse line blotting

Amplicons from cervical specimens typed by sequencing were used to establish the specificity of the probes. Immediately before use, amplification products (10 µl) were added to 140 µl 2 × SSPE-

0.1% SDS, denatured by boiling for 10 min, then placed on ice. After a brief spin, samples were brought to room temperature. The prepared membrane was placed in the miniblottedter, turned through 90° relative to its original position so that each slot crossed all the probes. The denatured products (130 µl) were added to the slots, the miniblottedter placed in a plastic bag with damp tissue and incubated for 1 h at 60°C to allow hybridisation. Samples were then removed by aspiration, the membrane removed from the blotter and washed twice with 2 × SSPE-0.5% SDS at 60°C. Probe-target hybrids were detected by enzyme-linked chemifluorescence using the ECF Signal Amplification Module (Amersham, UK). Briefly, membranes were rinsed in 0.3 M NaCl, 0.1 M Tris-HCl pH 7.5 and then incubated for 1 h with anti-fluorescein alkaline phosphatase conjugate (provided in the kit) diluted 1 in 5000 in 0.3 M NaCl, 0.1 M Tris-HCl pH 7.5 containing 0.5% BSA and washed three times in 0.3 M NaCl, 0.1 M Tris-HCl pH 7.5 containing 0.3% Tween 20. Membranes were then rinsed in 0.3 M NaCl, 0.1 M Tris-HCl pH 7.5. Signals were generated by the addition of 5 ml of detection reagent (provided in the kit), according to the manufacturer's instructions. Signals were detected on a Storm 860 System (Molecular Dynamics) using ImageQuant software.

### 2.7. Sequencing PCR products

An aliquot (1 µl) of the original amplification product was re-amplified with unlabelled GP5 + / GP6 + primers and the products (40 µl) purified using a Nucleon QC PCR clean up kit (Nucleon Biosciences, UK), according to the manufacturer's instructions. Re-amplification produced about 500 ng of target DNA with a low fluorescence which did not interfere with the sequencing methodology. DNA sequence was determined with the thermo sequenase dye terminator cycle sequencing pre-mix kit (Amersham, UK) according to the manufacturer's instructions. One strand of each product was subjected to sequencing with primer GP6 + on a 373A DNA sequencer (Applied Biosystems, UK). Resulting sequences were compared with the HPV database held on Genbank (Daresbury Laboratory, UK).

## 3. Results

### 3.1. Detection of HPV infection by conventional PCR

Cervical samples from 352 Sri Lankan women were tested for HPV by PCR using conventional GP5 + /GP6 + primers. Positive products were detected in 129 of the samples (36%) using agarose gels stained with ethidium bromide. These results show that for women under 26 years of age ( $n = 52$ ) the carriage rate of 47.7% was significantly greater ( $P = 0.0003$ ,  $\chi^2$  analysis) than the 28.6% carriage rate detected in women over 26 ( $n = 238$ ). There was no significant difference in the HPV carriage rate in commercial sex workers ( $n = 162$ ) and other women attending the STD clinic ( $n = 193$ ,  $P = 0.07$ , logistic regression with age as a confounding variable using SPSS). Differences in HPV infection rates according to number of sexual partners, parity, use of oral contraceptives, use of barrier contraceptives, and smoking habit were not statistically significant.

### 3.2. Development of HPV typing by reverse line blotting

Because the GP6 + primer was labelled at the 5' end with fluorescein positive PCR reactions could be typed directly using reverse line blotting. Initially all positive samples were reacted with a panel of 20 HPV probes comprising high risk types 16, 18, 31, 45, intermediate risk types 33, 35, 39, 51, 52, 56, 58, 59 and low risk types 6, 11, 42, 43, 53, 54, 66, 67. Probe specificity was confirmed with GP5 + /GP6 + products of known sequence. Under the hybridisation conditions used, the absolute specificity of 18 of the 20 probes was confirmed. However, weak one-way cross-reactions were detected with two HPV types. Samples confirmed as HPV-39 cross-hybridised weakly with the HPV-33 probe, similarly HPV-43 positive samples reacted with the HPV-45 probe. Given that both HPV-45 and HPV-33 reacted exclusively with their specific probe discrimination of HPV-45 from HPV-43 infection and HPV-33 from HPV-39 was straightforward.

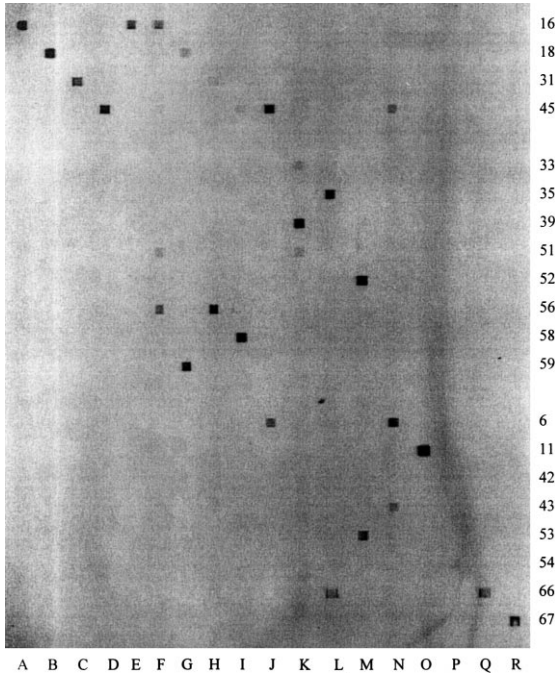


Fig. 2. An example of a reverse line blot showing single and mixed infections. The numbers indicate the probes (arranged horizontally in risk groups) and the letters represent the clinical samples (in vertical lines).

Reverse line blotting using an initial panel of 20 probes identified the HPV type(s) infecting 97 of the 129 PCR positive women. An example is shown in Fig. 2. Direct sequencing of amplicons non-reactive with these probes led to the additional design of probes for HPV types 62, 70, 72, han831 and CP8304. Using this expanded panel of probes only 10% of the HPV positive samples failed to type. Of the remaining women three were infected with the same HPV type which showed 80% sequence homology to HPV-72; additional genes are being sequenced to establish if this is a new type or a variant of HPV-72. The L1 gene sequence from one patient does not match with any type in Genbank data and may prove to be a new HPV type.

The different HPV types detected in Sri Lankan women are summarised in Fig. 3; the most important finding was that 57% of HPV infected women carried a high risk type. The multi-probe technique used enabled the ready detection of mixed infections with 18% of women shown to be infected with two types and 5% with three types; 59% of the mixed infections involved a high risk type.

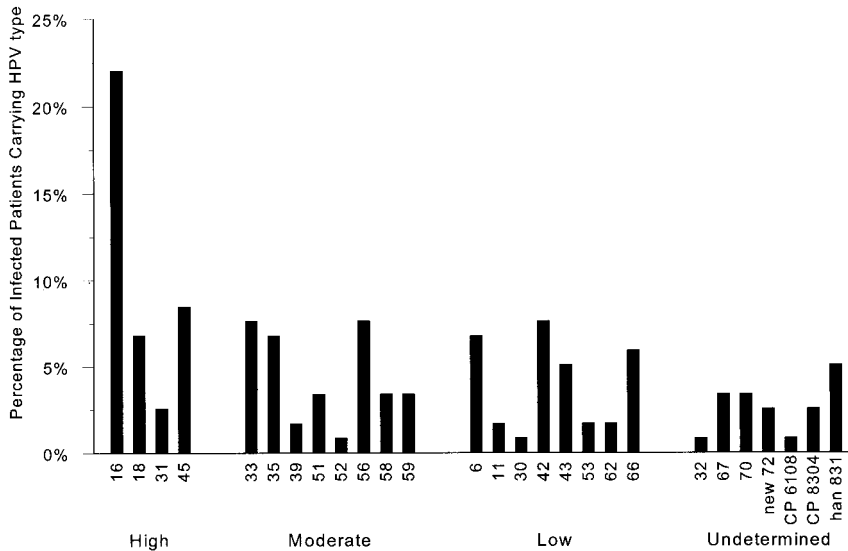


Fig. 3. Percentage of patients carrying each HPV type, with types grouped according to oncogenic risk.

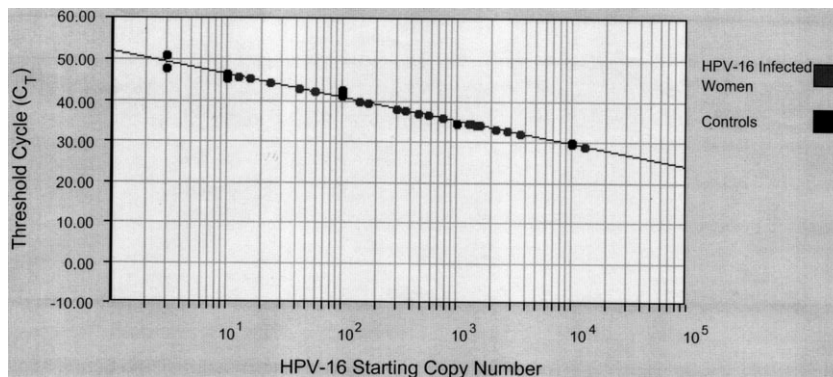


Fig. 4. SDS 7700 software-generated standard curve for HVP-16 showing standards (black) and clinical samples (grey). The starting quantity shows the number of copies of HPV-16 per PCR. The threshold cycle is the amplification cycle at which the fluorescence exceeds the threshold value, set on the basis of control reactions containing no template DNA.

### 3.3. Real-time detection of HPV positive amplicons

To facilitate the development of molecular beacon assays our initial studies focused on 21 women infected with HPV-16, detected by the above methods. Positive controls contained 3, 10,  $10^2$ ,  $10^3$  and  $10^4$  copies of cloned HPV-16 DNA. The buffers, dNTP concentration and DNA polymerase used were those recommended in the Taq-Man™ protocol (Perkin-Elmer, UK). Primer concentrations were varied between 1  $\mu$ M, 500, 300, 200 and 100 nM. Cycle conditions and fluorescent threshold values were also changed. The optimised conditions were shown to be primer concentration of 300 nM and a fluorescent threshold value of 0.027. The results of the experiment using these conditions are shown in Fig. 4. For cloned HPV-16 DNA,  $10^4$  copies were detected at 30 cycles,  $10^3$  at 35,  $10^2$  at 41, 10 at 46 and 3 copies at 50 cycles. Approximately 1/50 of the original clinical specimen is tested in the assay protocol; the results for the 21 HPV-16 positive women tested (Fig. 4) all fall well within the detection range of the assay. Comparable findings were obtained for HPV-18 (data not shown).

In order to compare molecular beacon assays with conventional PCR with primers GP5 + and 5'-fluorescein labelled GP6 +, 80 samples from Sri Lanka were tested in duplicate. Of the 40 confirmed positives, 11 were positive only by the

beacon detection system and five positive only by conventional PCR. Analysis established that samples detected by 45 cycles in the real-time fluorescent assay were strongly reactive on agarose gels whereas samples detected at 50 cycles were weakly reactive on ethidium bromide staining. The amplification products, which were 5'-labelled with fluorescein in the same way as the fluorescein-labelled GP6 + products, were readily typed using the multi probe hybridisation assay and enabled the detection of both single and mixed infections. HPV type CP6108, not represented in the probe panel, was easily detected.

## 4. Discussion

The present study developed a novel PCR for the detection of HPV infection based on molecular beacon primers which only fluoresce when incorporated into an amplicon. This enabled the PCR reaction to be monitored in real-time using an ABI Prism™ 7700 Sequence Detector. The method was shown to detect < 10 copies of the HPV genome. To the best of our knowledge, this is the first application of molecular beacon primers for the detection of microorganisms. Cervical samples from women attending an STD clinic in Sri Lanka were used to compare the sensitivity of the new method with conventional PCR and agarose gel detection, in order to estab-

lish the suitability of the method for use in large-scale field studies. To facilitate the rapid processing of samples we pelleted the HPV and infected cells by high-speed centrifugation and released the viral DNA by alkali hydrolysis. The hands-on time for processing 100 samples was around 3 h. Molecular beacon primers detected 11 infections missed by the agarose gel detection but missed 5 cases. All the reactions were extremely weak and the randomness of sampling inevitably leads to discrepancies in the results achieved by different HPV detection systems (Gravitt et al., 1998).

The conventional real-time TaqMan™ PCR assay uses unlabelled primers and a quenched fluorescent probe which is cleaved by the 5' exonuclease action of the polymerase. Given the hypervariability of papillomavirus genes this method requires individual probes for each HPV genotype. A recent study by Josefsson et al. (1999) developed a 5' exonuclease assay for HPV types 16, 18, 31, 33, and 35. Using different fluorescent dyes they demonstrated the feasibility of combining three HPV probes in a single PCR reaction. Nevertheless this improved probe method would still require a multiplicity of PCR reactions on every patient for population studies. Thus we would require around ten reactions (30 probes) to detect the different HPV types found in the cervical samples from 352 Sri Lankan women. In contrast the molecular beacon primers we developed detect all HPV types in a single PCR reaction producing a fluorescently labelled amplicon which can be directly typed by reverse-line blotting. A comparable reverse line blotting was developed by Gravitt et al. (1998). This uses biotinylated MY09/MY11 primers detecting amplification products with a single strip reaction for each patient. An important gain is that the method readily detects mixed HPV infections. A significant advantage of our molecular beacon approach for population studies and high-throughput HPV testing is that only confirmed HPV infections are typed in a reaction which tests 45 amplicons simultaneously. Further, molecular beacon primers are capable of detecting all known HPV types whereas screening for HPV by line blotting misses any infection caused by genotypes not included in the cocktail of 27 probes used. In

our study this cocktail would have missed 32 of the 129 infections detected and precludes the search for any novel HPV types infecting these women. Importantly, molecular beacon amplicons which are not typed by the current probe panel can be typed by sequencing after a simple dilution and re-amplification step.

Although the L1-based primer sets GP5+ / GP6+ and MY09/MY11 (Manos et al., 1989) are used widely in HPV studies, the efficiency of amplification for the different genotypes varies according to the number of mismatches present (Qu et al., 1997). To overcome this, Gravitt et al., (2000) have recently designed primer pools, based on MY09/MY11 primers, which enable efficient amplification of more HPV types. It would be possible to tag all the primers in either pool with the molecular beacon sequence. For economic reasons, and to minimise the risk of primer–primer interactions, it would be preferable to label the five sense primers rather than the 13 antisense primers. A novel universal primer set, designated SPF1 and SPF2, was shown to detect HPV DNA in 100% of the cervical carcinoma specimens tested (Kleter et al., 1998). A major advantage is that only the two SPF primers would require tagging. The important limitation lies in HPV typing given that the 65 bp amplification product of the L1 gene restricts both probe design and length of sequencable DNA.

PCR with fluorescein-labelled primers and reverse line blotting were used to screen 352 women attending a STD clinic in Sri Lanka. Papillomavirus DNA was detected in 129 women with 57% of these infected with high risk types. Reverse line blotting enabled the ready detection of mixed infections with 21 infections containing two or more HPV types. Mixed infections are detected in all grades of CIN (Kalantari et al., 1997) and have been associated with increasing severity of disease (Becker et al., 1994). The HPV carriage rate of 52.3% in young women (14–23 years of age) attending this clinic was higher than in many other studies (Cuzick et al., 1998), perhaps reflecting the recent increase in women working in the sex industry. Despite the high prevalence of HPV infection, little cytopathology was detected with 95% of the infected women having no evidence of

cervical dyskaryosis. Presumably, most infections were detected at an early stage and follow-up studies are needed to establish the outcome of infection in these women. Currently cervical cancer accounts for 25.4% of all cancers in Sri Lankan women. The spread of potentially oncogenic HPV infection into the community combined with the lack of a nation-wide cytology programme would lead to a significant increase of this cancer.

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