JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 2001, p. 3204–3212 Vol. 39, No. 9
0095-1137/01/$04.00+0 DOI: 10.1128/JCM.39.9.3204–3212.2001

Novel Method for Detection, Typing, and Quantification of Human Papillomaviruses in Clinical Samples

KEITH W. HART,[1]* O. MARTIN WILLIAMS,[1] NICOLA THELWELL,[2] ALISON N. FIANDER,[3] TOM BROWN,[4] LESEK K. BORYSIEWICZ,[1]† AND COLIN M. GELDER[1]

Section of Infection and Immunity, Department of Medicine, University of Wales College of Medicine, Heath Park, Cardiff CF14 4XX,[1] Department of Obstetrics and Gynaecology, Llandough Hospital & University Hospital of Wales, Cardiff CF4 4XX,[3] Oswel Research Products, Ltd., University of Southampton, Southampton SO16 7PX,[2] and Department of Chemistry, University of Southampton, Highfield, Southampton SO17 1BJ,[4] United Kingdom

Received 2 April 2001/Returned for modification 20 May 2001/Accepted 16 July 2001

We report the development of a novel detection and typing methodology for human papillomaviruses (HPV) based on real-time PCR with the self-probing fluorescent primers known as Scorpions. This technique is quick, simple, specific, sensitive, and capable of estimating viral load per cell. We report the results of over 100 typing reactions performed on cell lines, biopsies, and cervical cytobrush samples which, when compared to the current reference HPV detection and typing technique, present a κ value of 0.89. We further report preliminary data suggesting a relationship between viral load per cell and grade of cervical disease.

Cervical cancer is the second most frequent cause of death from cancer in women worldwide (16). Cervical screening programs reduce the incidence of cervical cancer (18); however, 50% of invasive cervical cancers arise in women screened with existing cytological methodologies (5). In recent years it has been established that a subset of human papillomaviruses (HPV) is associated with cervical cancer, and it is estimated that HPV DNA is present in over 99% of these cancers (22). There are currently 84 types of HPV, approximately 30 of which infect the genital tract (17). The infecting HPV type, the viral load, and the integration state of the HPV genome are known to have profound implications for patient prognosis (10, 19, 28). Thus, HPV detection and typing techniques have been proposed as an adjunct to, or a replacement for, the current cytological screening regime (3, 4, 12, 25). Clearly the success of such strategies will depend on the development of rapid, reliable, sensitive, and specific HPV detection methods applicable in the clinical setting.

Currently, there are eight main approaches to the detection and typing of HPV, all of which display advantages and disadvantages depending on their application (for reviews, see references 7, 15, and 21). Despite recent innovations (6, 14), no single technique performs optimally in both clinical and research settings.

We report a new technique for HPV typing that we have named viral evaluation using self-probing amplicons (VESPA). VESPA is a real-time PCR-based technique that utilizes self-probing amplicon primers known as Scorpions (24). This methodology is well suited to HPV detection, since it is simple to perform, rapid, highly specific (20), and reproducible and has the potential to measure viral load. We report typing results for 108 samples, including cell lines, cervical cytobrush samples, and biopsies, performed using both VESPA and the current reference technique, PCR-enzyme immunoassay (EIA) (8). We also present preliminary viral load data for 16 clinically defined HPV-16-positive samples.

* Corresponding author. Mailing address: Section of Infection and Immunity, Department of Medicine, University of Wales College of Medicine, Heath Park, Cardiff CF14 4XX, United Kingdom. Phone: (029) 20745004. Fax: (029) 20745003. E-mail: HartKW@cf.ac.uk.

† Present address: Imperial College School of Medicine, London SW7 2AZ, United Kingdom.

MATERIALS AND METHODS

Cell lines. The HeLa, Caski, and SiHa cell lines were a kind gift from Steve Man, University of Wales College of Medicine, Cardiff, United Kingdom.

Clinical samples. Patients either were recruited during routine colposcopy clinics at Llandough Hospital, Cardiff, United Kingdom, or were part of an Medical Research Council field study in The Gambia. Informed consent was obtained from all subjects. Cervical samples were collected using conical cytobrushes and transported in 0.5 ml of Digene transport medium (Silver Spring, Md.). Samples taken in the United Kingdom were stored at 4°C for up to 24 h before processing. Samples collected in The Gambia were stored frozen in liquid nitrogen, shipped to the United Kingdom on dry ice, and processed within 1 month.

DNA purification. DNA was purified from cervical cytobrush samples by a simple modification of the freeze-thaw method of Jacobs and coworkers (8). In brief, epithelial cells obtained from the cytobrush samples were pelleted by centrifugation, resuspended in 1 ml of 10 mM Tris (pH 7.4), and frozen at −70°C for 24 h. A 100-μl aliquot was thawed, boiled for 10 min, chilled on ice, and spun in a microcentrifuge (13,000 rpm; MSE Microcentaur) for 3 min, and the supernatant was decanted and stored.

DNA was extracted from biopsy material using a modification of the above technique, in which samples were incubated in 1 ml of 10 mM Tris HCl (pH 7.4) containing proteinase K (Sigma) (10 mg/ml) for 1 h at 56°C before being boiled.

DNA was purified from cell lines by resuspension of cells in 640 μl of nuclear lysis buffer (10 mM Tris HCl, 0.4 M NaCl, 2 mM ethylene diamine tetraacetate [pH 8.0], 10% sodium dodecyl sulfate), 100 μl of 6 M NaCl, and 740 μl of chloroform. The solution was thoroughly mixed and centrifuged, and the top phase was extracted. DNA was precipitated by the addition of 1 ml of 95% ethanol and pelleted by centrifugation. The pellet was washed twice with 70% ethanol, dried in a rotary evaporator, and resuspended in 500 μl of deionized water.

PCR-EIA. PCR-EIA was performed using the technique of Jacobs et al., as previously described (8).

VESPA. PCR amplification of 1 μl of DNA solution was performed using 0.5 μM Scorpion primer (see Table 2) and 0.5 μM $GP5^+$ reverse primer (8) in a total reaction volume of 10 μl. Reactions were performed using a Light Cycler (Bio/Gene, Kimbolton, Cambridgeshire, PE18 0NJ, United Kingdom, or Roche Diagnostics, Ltd., Lewes, East Sussex BN7 1LG, United Kingdom) and run for 100 cycles under the following cycling parameters: 96°C for 1 s, 40°C for 5 s, and 72°C for 1 s. The reaction mixture conditions were as follows: 200 μM deoxynucleoside triphosphates, 4 mM $MgCl_2$, 50 mM Tris HCl (pH 8.9), 10 mM ammonium

sulfate, 0.1% Tween 20, bovine serum albumin (250 ng/μl), and 0.5 U of Taq polymerase (Advanced Biotechnologies, Epsom, Surrey, United Kingdom)/μl. Fluorescence was detected in channel one (530 nm) at 40°C. Control PCRs were carried out as above, but reaction mixtures included 1 μl of SYBR Gold (Bio/Gene). Scorpion control reaction mixtures (negative controls) contained 1 μl of H_2O in place of DNA. All primers were synthesized by Oswel Research Products, Southampton SO16 7PX, United Kingdom. Degenerate HPV detection was performed using DNA preamplified with a tailed primer (Table 1) and GP5$^+$ under conditions previously described (8). One microliter of this reaction mixture was then added to the VESPA reaction mixture containing both degenerate Scorpion primers (Table 1) and under the same conditions as those described above.

Primer design. Table 1 shows the sequences of the 10 Scorpion typing primers used in this study. The primer sequence of each Scorpion is type specific and is located at the same sequence position as that of the GP6$^+$ primer of Jacobs et al.(8). Scorpion probe sequences were designed by aligning the L1 open reading frames (ORFs) of 20 common HPV types (HPV-6, -11, -16, -18, -31, -33, -35, -39, -40, -42, -43, -44, -45, -51, -52, -56, -58, -59, -66, and -68) (http://hpv-web.lanl.gov). The area of greatest sequence variation adjacent to the GP6$^+$ primer binding site was selected as the probe target binding site. The probe sequence of these primers was checked against 70 common papillomavirus sequences, and no significant homology was found. The reverse primer target sequence is the GP5$^+$ sequence of Jacobs and coworkers (8). Fig. 1a and 1b show the structure and mechanism of Scorpion primers.

Degenerate HPV primers were also designed around the GP6$^+$ primer sequence. A preamplification reaction was run under the conditions described in reference 8, with a primer containing a unique designer tail (Fig. 1c). After the second round of amplification, this tail became incorporated into the amplicon, creating a single target site for a specific degenerate Scorpion primer. The Scorpion probe was designed to detect the GP6$^+$ section of the primer. The background due to the detection of primer dimer in this system, however, was unacceptable. We therefore designed a Scorpion that overlapped by three bases into the freshly synthesized amplicon. Two such Scorpions were required to provide complete coverage of the 40 most common HPV sequences.

RESULTS

Typing primer validation. The HPV-16 and -18 primers were tested for specificity using reference cell lines with integrated HPV DNA. The Caski cell line contains 60 to 600 copies of the HPV-16 ORF per cell (13), and the HeLa cell line contains 10 to 50 copies of the HPV-18 L1 ORF (13). Figure 2a shows the results of separate PCRs using the Sc16 Scorpion primer (designed for detection of HPV-16 DNA), DNA extracted from the HPV-16-positive Caski cell line, DNA from the HPV-18-positive HeLa cell line, and a negative control (no DNA). A significant increase in fluorescence was detected only with the HPV-16-containing Caski DNA.

Figure 2b shows a similar experiment with the HPV-18-positive HeLa cell line. Here, significant fluorescence was detected only with the Sc18 Scorpion. These primers were then used to detect HPV-16 and -18 in clinical samples previously typed by PCR-EIA (Fig. 3) and extended to enable detection of HPV-6, -11, -31, -33, -39, -51, and -56. Since cell lines containing these HPV types are not commercially available, primer specificity was validated using clinical samples previously tested by PCR-EIA. Figure 3 shows positive results from typing reactions for HPV-6, -11, -16, -18, -31, -33, -39, and -51. In each experiment, cross-reactivity with all other Scorpions was investigated in separate reactions and found to be negligible. These results are not included for the sake of clarity but are available on request.

Viral load determination. A theoretical advantage of VESPA is its ability to determine viral load. Shown in Fig. 4a are the results of Sc16 typing reactions performed using a dilution series of the SiHa cell line (one to two copies of HPV-16 per

TABLE 1. Sequences of VESPA primers[a]

Primer name and type	Sequence
Typing primers	
HPV-6 Sc6	5′-FAM c c g c g g A T A A A G A G T A C A T G C G T c c g c g g MR HEG G A A A A A T T G T A A A T C A T A C T C -3′
HPV-11 Sc11	5′-FAM c c g c g g C A G A T T A T A A G G A A T A C A T G C c c g c g g MR HEG G A A A A A T T G T A A A T C A A A C T C -3′
HPV-16 Sc16	5′-FAM c c g c g g A G T A C C T A C G A C A T G G G c c g c g g MR HEG G A A A A A T T G T A A A T C A T A T T C -3′
HPV-18 Sc18	5′-FAM c c g c g g c A G C A G T A T A G C A G A C A T G g c c g c g g MR HEG G A A A A A T T G T G C A A A T C A T A T T C -3′
HPV-31 Sc31	5′-FAM c c c g c g g A G T A T T T A A G A C A T G G T G C c g c g g MR HEG G A A A T T A T T G T A A A T C A A A T T C -3′
HPV-33 Sc33	5′-FAM c c c g A g g A A G A A T A T A T A A G A C A T G T T G C c T c g g MR HEG G A A A A A C A T G T A G A T C A T A T T C -3′
HPV-39 Sc39	5′-FAM c c c g c g g A A T A T A C C A G G C A C G T G C c g c g g MR HEG G A A A T A T T G T A A A T C A T A C T C -3′
HPV-45 Sc45	5′-FAM c c c g c g g G C A G T A T A G A C A T G T G G C c g c g g MR HEG G A A A A A T T G T A A A T C A T A T T C -3′
HPV-51 Sc51	5′-FAM c c c g c g g G C A A T A T A T T A G G C A T G G G C c g c g g MR HEG G A A A A A T T G T G C A A T T C A T A C T C -3′
HPV-56 Sc56	5′-FAM c c g c g g T C A G T A C C T A G G T A G A T A C T A C -3′ MR HEG G A A A A A C A A A T T G T A A A C C C A T A T T C -3′
Reverse primer GP5$^+$	5′ T T T G T T A C T G T G G T A G A T A C T A A C -3′
Degenerate HPV primers	
ScDGi	5′-FAM c c g c g g G A A G A A T A T G A T T T A C A C c g c g g MR HEG G T G G A A A C A T G C A T G G C G A C -3′
ScDGii	5′-FAM c c g c g g G A G G A A T A T G A T T T A C A C c g c g g MR HEG G T G G A A A C A T G C A T G G C G A C -3′
Tailed GP6$^+$	5′ A T G T G G A A A C A T G C A T G G C G A C A T G A A A A A T A A A C T A A A T C A T A T C T -3′
Beta-globin detection primers	
ScBG	5′-FAM c c g c g g G A T G G T G T C T G T T T G A G c c g g c g g MR HEG A C A C A A C T G T G T T C A C T A G C -3′
Reverse primer	5′ G A A A C C C A A G A G T C T T C T C T -3′

[a] All Scorpion primers were purified by double high-performance liquid chromatography. Standard primers were purified by gel filtration. Primers are designed to detect the HPV L1 gene. Primer locations vary between types but are positioned approximately between 6,600 and 6,750 bp. Scorpion primer and probe sequences are shown in uppercase letters. Scorpion stem sequences are shown in lowercase. FAM, fluorophore (6-carboxyfluorescein); MR, fluorescence quencher (methyl red); HEG, PCR blocker (hexethyl glycol).

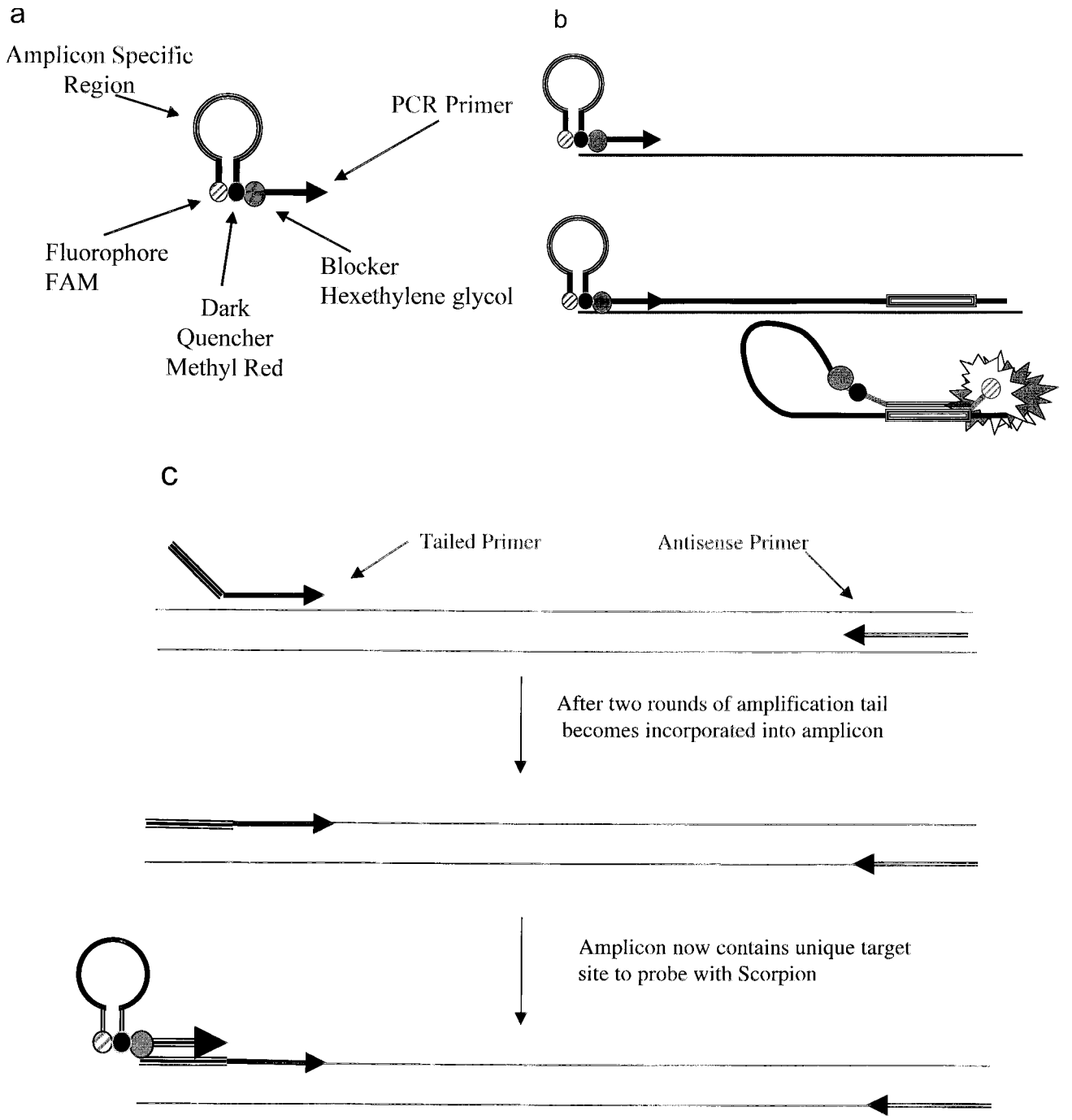

FIG. 1. (a) Structure of Scorpion primer. (b) Mode of action of Scorpion primers. The Scorpion primer consists of a conventional PCR primer attached to a looped tail. The tail consists of a stem region in which the DNA is self complementary and a single-stranded loop section, or probe, contains a sequence that is complementary to a section up to 80 bp downstream of the primer binding site. The stem section keeps a fluorophore and dark quencher in close proximity. If the primer finds a binding site and amplification proceeds, a probe binding site is produced. When the probe binds to this site, the loop is opened thus removing the fluorophore from the dark quencher. An increase in fluorescence results. A PCR blocker prevents read-through of the looped tail in subsequent rounds of amplification. (c) Mode of action of degenerate Scorpions. With a tailed primer containing a designer sequence in the tail, it is possible to introduce a unique target sequence into the amplicon after the second round of amplification. This sequence can then provide the sole target for a single Scorpion mixture (ScDGi and ScDGii) theoretically capable of detecting over 40 HPV types.

cell) (13). The dilution series from 50,000 to 500 HPV viral copies was clearly distinguishable, and the signal for HPV-16 remained positive down to a single copy of HPV-16 DNA.

To explore the possibility of establishing a quantitative HPV assay capable of producing an estimate of viral load per cell, a Scorpion primer was designed to detect (human) beta-globin DNA (ScBG) (Table 1). The beta-globin gene is a common test gene included in many PCR-based assays for infectious

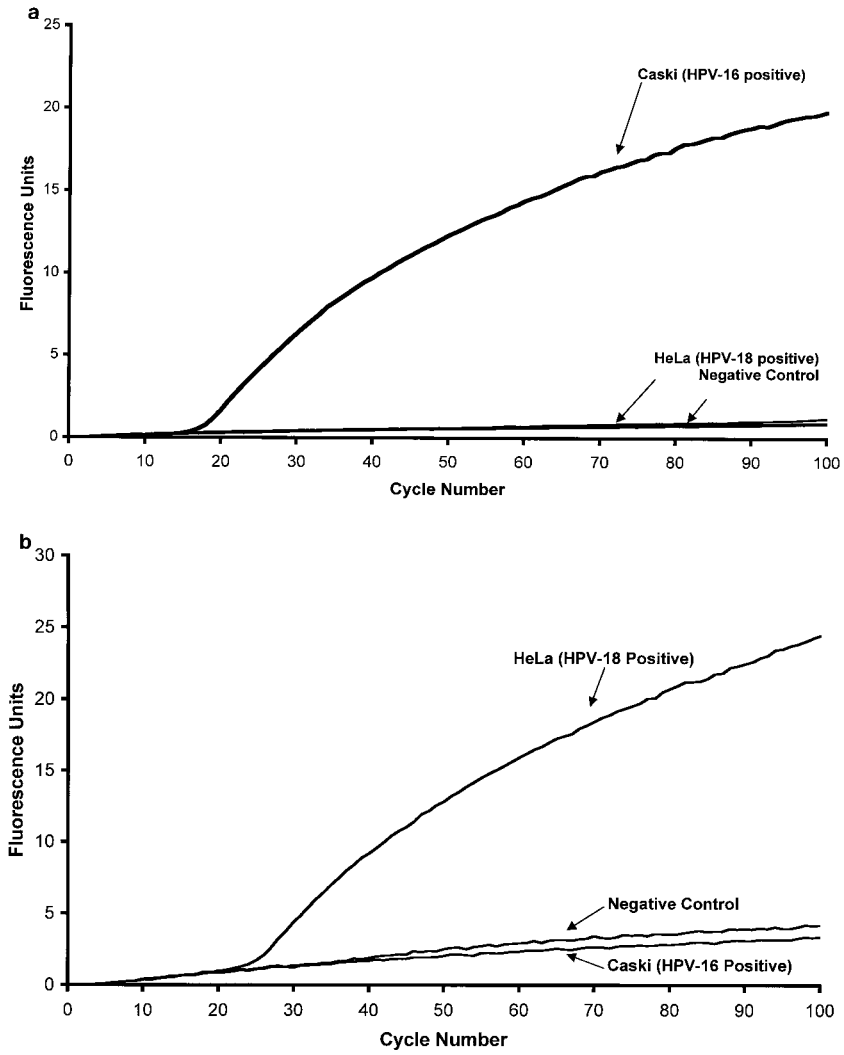

FIG. 2. (a) HPV-16 detection. The results of HPV typing reactions using the Scorpion primers specific for HPV-16 (Sc16), HPV-18 (Sc18), a negative control (no DNA), and DNA extracted from a HPV-16-specific cell line (Caski) are shown. (b) The results of HPV typing reactions using the Scorpion primers specific for HPV-16 (Sc16), HPV-18 (Sc18), a negative control (no DNA), and DNA extracted from an HPV-18-specific cell line (HeLa) are shown.

disease to control for PCR viability. Here we have extended its use to that of a housekeeping gene, as a measure of the amount of human DNA that was sampled. Figure 4b shows the results of an experiment conducted using ScBG over the same dilution series of SiHa cells used for Sc16. The fluorescent signal remained positive down to a single cell and is quantitative at and above this level.

Since we had a quantitative measure of both viral copy number and cell copy number, as reflected by the number of copies of the beta-globin gene, we calculated the number of viral copies per cell. A range of copy numbers per cell may be determined from a cell line containing a fixed copy number by calculating the Sc16/ScBG fluorescence ratio for two different dilutions, e.g., the Sc16 F value (Sc16 F_{max} − Sc16 F_{min}) for 5,000 HPV-16 copies can be divided by that for the ScBG F value (ScBG F_{max} − ScBG F_{min}) for 100 beta-globin gene copies to obtain a value for a notional cell line containing 50 copies per cell (5,000/100). Figure 5 shows a plot of the ratio of the fluorescence produced by Sc16 and ScBG in the SiHa dilution series (measured in relative virus units) against the logarithm of the viral copy number per cell. The relationship between the fluorescence ratio of Sc16 to ScBG and the logarithm of the viral copy number per cell is broadly linear over 4 orders of magnitude.

Shown in Table 2 are the results of applying this viral load determination technique to 16 clinical samples previously found to be HPV-16 positive by PCR-EIA. For each sample, the fluorescence value for HPV-16 was divided by the fluorescence value for human genomic DNA and converted to average copies per cell with the standard curve shown in Fig. 5 and Graphpad Prism software, version 2.0 (Intuitive Software for Science, San Diego, Calif.). The most striking finding from these experiments is that the four cervical smears with normal cytology (samples 1 to 3 and 5) have low viral loads (below the detection limit of VESPA). The other sample with a low viral load (for HPV-16) but significant neoplasia is coinfected with HPV-6 and HPV-39 (sample 4).

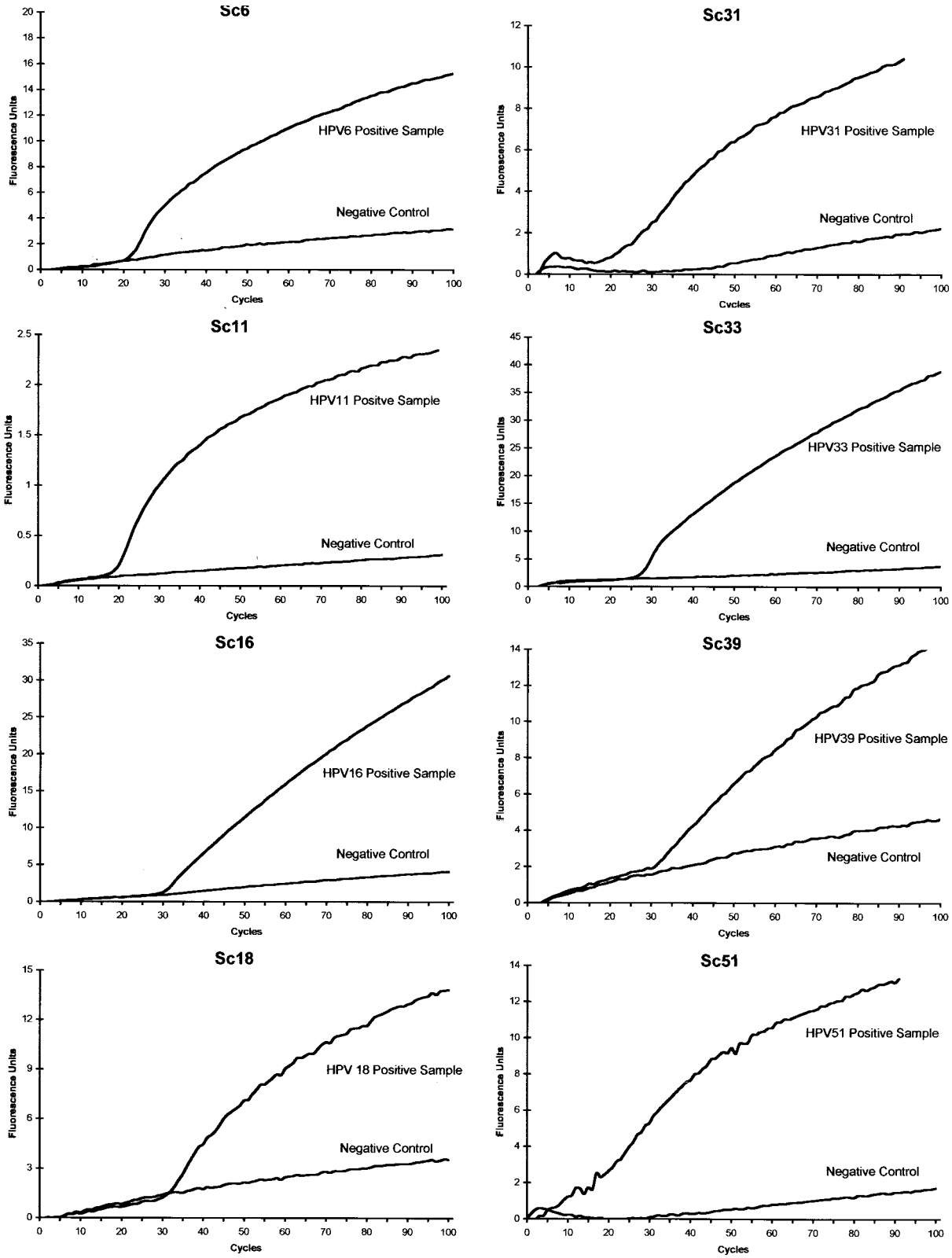

FIG. 3. Examples of positive traces produced by VESPA. The results of HPV typing experiments using clinical samples previously typed using PCR-EIA are shown. Primers specific for HPV-6 (Sc6), HPV-11 (Sc11), HPV-16 (Sc16), HPV-18 (Sc18), HPV-31 (Sc31), HPV-33 (Sc33), HPV-39 (Sc39), and HPV-51 (Sc51) are shown.

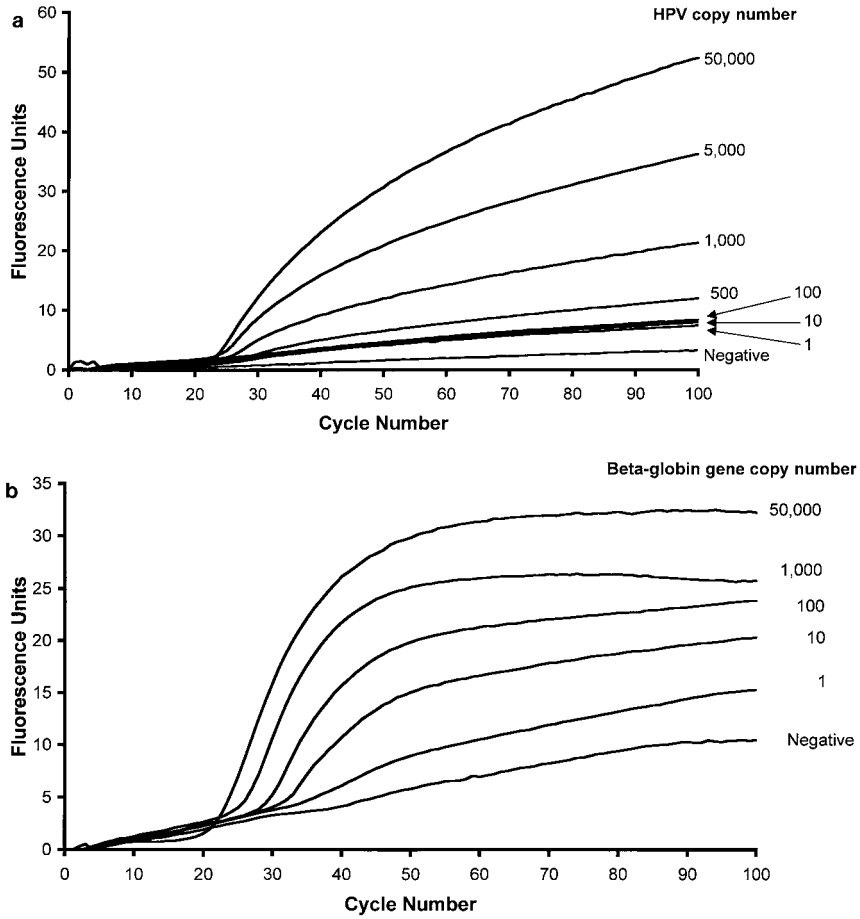

FIG. 4. Demonstration of quantitative nature of VESPA. (a) HPV-16 dilution series with Sc16. A dilution series of SiHa cells was made from 50,000 cells per reaction mixture to 1 cell per reaction mixture. (b) Beta-globin gene detection using ScBG and the dilution series described in the legend to panel a.

Comparison to PCR-EIA. To test the suitability of VESPA for HPV typing of cervical smears in the clinical setting, the Scorpion primers were used to test 108 samples previously HPV typed by PCR-EIA (8). The test was performed blinded to the PCR-EIA result, and each sample was tested with all Scorpions. To directly compare the two techniques, DNA extraction was performed using the freeze-thaw method described by Jacobs and colleagues (8). This technique is suboptimal for PCR amplification using Scorpion primers (see discussion). The results of these experiments are shown in Table 3. Selected positive sample types have subsequently been confirmed by direct sequencing (data not shown). The overall concordance between VESPA and PCR-EIA is 94% with a κ value of 0.89, indicating good agreement (1). Of 108 samples, VESPA failed to detect five incidences of HPV-16 and one of HPV-18 (see Discussion). There were no false positives.

Screening using VESPA. To expand the capability of VESPA for use in HPV screening, we designed a degenerate HPV Scorpion mixture for use in conjunction with a tailed general primer (Table 1). By utilizing a tailed primer, we were able to introduce a consensus site that enables a single Scorpion to recognize many different HPV amplicons (Fig. 1c). This is a two-step procedure that can theoretically detect over 40 different HPV types. Figure 6 demonstrates the ability of the degenerate Scorpion mixture to detect HPV-6, -16, and -18.

DISCUSSION

We report a novel real-time PCR-based technique for the detection, typing, and quantification of HPV in clinical samples which we have termed VESPA. We believe VESPA has several advantages over existing HPV detection and typing methods in terms of its speed, ease of use, sensitivity, specificity, and capability to provide quantitative information about viral load.

When compared to the two front-line HPV detection and typing methodologies, Hybrid Capture II and PCR-EIA, VESPA is technically less demanding and produces results more rapidly.

The sensitivity of VESPA in cell lines is at least 2 orders of magnitude higher than that reported by Digene (5,000 copies) for Hybrid Capture II, which has been approved by the Federal Drug Administration for HPV screening. VESPA has sensitivity comparable to that of other previously published HPV detection techniques, including PCR-EIA (10 to 200 viral copies), in which the detection limit is calculated with enriched control templates against a low background of genomic DNA

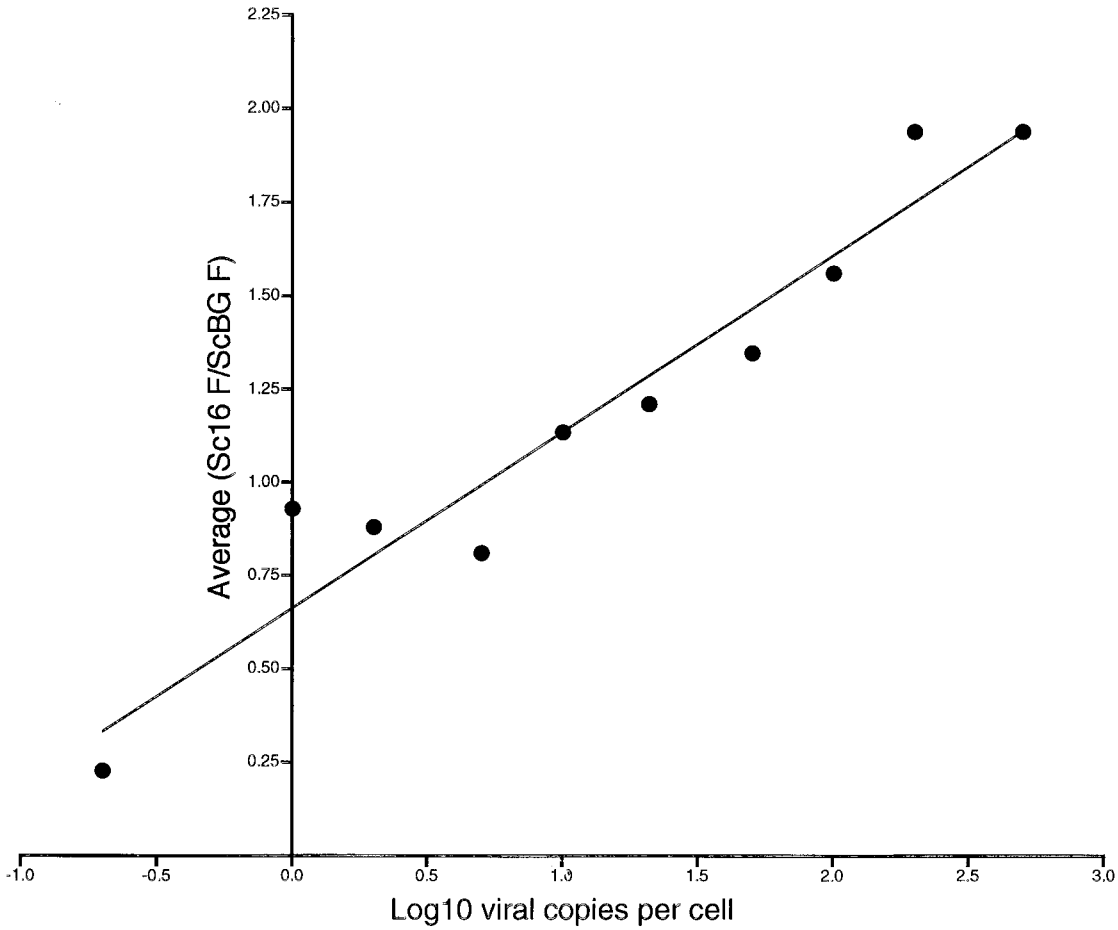

FIG. 5. Estimation of viral load Graph. Data were calculated as described in the text.

(8, 19, 28). Although the exact viral threshold for immediate risk of carcinogenesis is controversial (and may vary with HPV type and between individual patients) the available evidence suggests that it is likely to be above VESPA's lower detection limit (19, 28).

VESPA is type specific and compares favorably with Hybrid Capture, in which the probes for many different HPV types are multiplexed and in which samples are categorized as low-risk and high-risk HPV types in two reactions (21, 26). The fact that Scorpion primers may be multiplexed has been demonstrated by us (data not shown) and others (20), and thus VESPA could also be used in this manner as a primary cervical screen, as is currently being investigated for Hybrid Capture II. Screening with multiplexed probes, however, can lead to probe cross-reactivity (21). We intend to validate the utility of VESPA as a primary screen in a large prospective study using degenerate Scorpion probes (discussed below).

The HPV typing achieved using VESPA correlates well in our hands with data obtained using PCR-EIA ($\kappa = 0.89$), the most-established HPV typing method. Indeed, a similar concordance figure was achieved when PCR-EIA was performed on identical samples by different reference laboratories (9).

There are several explanations for the small number of discrepancies observed between VESPA and PCR-EIA. First, the results produced by VESPA might be false negatives. The

TABLE 2. HPV-16 viral load in clinically defined samples[a]

Sample	HPV type(s)	Disease grade	VL ratio (RVU)	Avg no. of viral copies/cell
2885C	16	Normal	−0.3508	N/A
2503F	16	Normal	−0.1204	N/A
0155H	16	Normal	−0.1007	N/A
LD20	6, 16, 39	CIN2	−0.0242	N/A
0119D	16	Normal	−0.0166	N/A
SJ	16	Ca vagina	0.0095	0.042
LD49	16	CIN2-3	0.0331	0.047
AD	16	CIN2-3	0.0550	0.052
LD19	16	CIN2	0.0697	0.056
LD15	16	CIN3	0.0750	0.057
LD25	16, 31	CIN3	0.0786	0.058
LD24	16	CaCx	0.1641	0.088
LD50	16	CIN3	0.4945	0.441
LD31	16	CIN2-3	0.5965	0.724
LD45	16	CIN2-3	0.7601	1.603
0093A	16	CIN2-3	0.8749	2.805

[a] All samples were purified from cervical cytobrush samples. The disease grade was determined by cytology. The average number of viral copies per cell is estimated using the standard curve shown in Fig. 5. We assume two copies of the HPV-16 L1 gene and two copies of the beta-globin gene per SiHa cell. CIN, cervical intraepithelial neoplasia; Ca vagina, cancer of the vagina; CaCx, cancer of the cervix; NA, not applicable (i.e., below assay detection level). VL ratio (viral load) is measured in relative virus units (RVU) as described in the text.

TABLE 3. Comparison of PCR-EIA[a]

Origin	HPV type(s)	No. of positive samples (n = 108) by:	
		PCR-EIA	VESPA
Cell lines (n = 3)	16	2	2
	18	1	1
Laryngeal biopsy (n = 3)	6	1	1
	11	2	2
Cervical sample (n = 102)	11	4	4
	16	15	10
	18	6	5
	31	2	2
	33	4	4
	39	2	2
	51	3	3
	56	3	3
	16, 31	2	2
	16, 39	1	1
	33, 51	1	1
	6, 16, 39	1	1
	Negative	58	64

[a] The κ value (0.89) was calculated as described in reference 8.

presence of PCR inhibitors in cervical samples has been previously reported (11). Using a standard chloroform extraction-based method of DNA purification, we have demonstrated that it is possible to improve the signal produced by VESPA that is only weakly positive to detect HPV in cervical smear samples when purified by the freeze-thaw technique. We have used the less-efficient freeze-thaw DNA extraction method in this study, since this method is recommended for PCR-EIA and we wished to compare PCR-EIA and VESPA using identical DNA samples.

Second, the results produced by PCR-EIA might be false positives. There is evidence from a study comparing results from several different laboratories that PCR-EIA is prone to the occasional false positive (9).

Third, the samples may be positive for HPV but have an intratypic HPV variant containing polymorphism within the probe binding site. Such samples will not be overlooked, since all samples are first screened (currently by PCR-EIA) for HPV positivity. Scorpion probes discriminate between sequences on the basis of a single base change (20), whereas PCR-EIA probes are more tolerant of sequence variation (23). In this regard it may be relevant that five of six discordant samples were obtained from patients from The Gambia, where samples are likely to show more variation within the probe binding site (27). This increased specificity may require the inclusion of extra Scorpions to confer complete coverage while also providing the ability to investigate the relative risk associated with sequence variants. We are currently sequencing these samples.

VESPA has the potential to estimate viral load. There is increasing evidence that viral load is a critical determinant in patient prognosis (10, 19, 28). Indeed, some authors have suggested that the high-risk types may be more potent, not due to the increased oncogenicity of their transforming proteins, for example, but simply because they proliferate more efficiently, overwhelming the immune response (2). Our preliminary viral load data suggest that the presence of cervical neoplasia might be related to the viral load. Four HPV-16-positive samples (by PCR-EIA), among our cohort of 108 and obtained from subjects with normal cervical cytology, have undetectably low viral loads by VESPA. The only subject with significant neoplasia and a low viral load was coinfected with HPV-6 and -39. It is perhaps surprising that no sample contains an apparent viral load of >3 per cell, since other groups have reported viral loads estimated to be an order of magnitude higher than this (29). However, the range of values quoted varies enormously (in the range of 10^{-5} to 10^{3} genome copies per cell) and are often produced using semi-quantitative techniques. It is also important to consider, when analyzing viral load data, that values are an average summed over many cells, a large proportion of which may not be infected, and that the viral DNA may have become integrated, disrupting or deleting the probe target site. The fact that high viral loads are associated with more-severe disease may be because severe disease produces high viral loads rather than vice versa. We intend to undertake a large prospective study of the relationship between clinical status and virus type, load, and integration status using VESPA.

The major application of VESPA is likely to be in probing

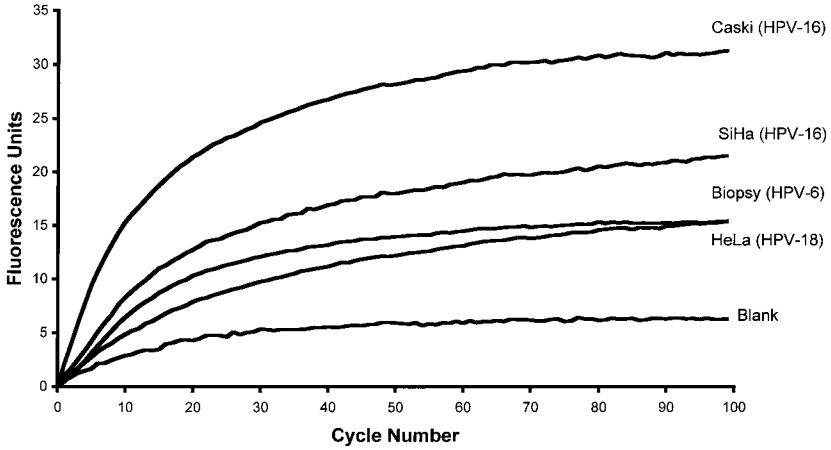

FIG. 6. Degenerate HPV detection using VESPA. Reactions were performed as described in the text using samples preamplified with a tailed primer to introduce a unique primer target site.

the molecular etiology of HPV-associated disease rather than in the primary clinical evaluation of cervical neoplasia. However, we have designed a degenerate Scorpion mixture that is theoretically capable of detecting 40 of the most common HPV types and demonstrably capable of detecting HPV-6, -11, and -18 (Fig. 6). Interestingly, two of these samples, when assessed by PCR-EIA, were barely visible after agarose gel electrophoresis with ethidium bromide staining, suggesting that this approach produces the expected improvement in sensitivity. Thus, VESPA may also find an application in screening for HPV.

In conclusion, we have developed a novel method for the characterization of human papillomavirus infection. This new method is quicker (<1 h), more specific (single-base discrimination), and less laborious (single step) than currently available techniques and, unlike most techniques, is capable of estimating viral load. Future work will concentrate on increasing the armory of Scorpions to cover more HPV types, to include probes capable of assessing the physical state of the HPV genome and to modify VESPA for use in microtiter plate format.

ACKNOWLEDGMENTS

We are indebted to all the women who consented to take part in this study. We are grateful to Keith McAdam, Gijs Walraven, Beryl West, Caroline Scherf, and all the staff of the MRC Field Station, Farafenni, The Gambia.

C.M.G. is a Wellcome Senior Fellow in Clinical Research; O.M.W. is a Wellcome Research Training Fellow. This research was supported by an MRC project grant (no. G9901202).

REFERENCES

1. **Armitage, P., and G. Berry.** Statistical methods in medical research, 3rd ed. Blackwell Scientific Publications, Oxford, United Kingdom.
2. **Bible, J. M., C. Mant, J. M. Best, B. Kell, W. G. Starkey, K. Shanti Raju, P. Seed, C. Biswas, P. Muir, J. E. Banatvala, and J. Cason.** 2000. Cervical lesions are associated with human papillomavirus type 16 intratypic variants that have high transcriptional activity and increased usage of common mammalian codons. J. Gen. Virol. **81:**1517–1527.
3. **Costa, S., M. Sideri, K. Syrjanen, P. Terzano, M. De Nuzzo, P. De Simone, P. Cristiani, A. C. Finarelli, A. Bovicelli, A. Zamparelli, and L. Bovicelli.** 2000. Combined Pap smear, cervicography and HPV DNA testing in the detection of cervical intraepithelial neoplasia and cancer. Acta Cytol. **44:** 310–318.
4. **Cuzick, J.** 2000. Human papillomavirus testing for primary cervical cancer screening. JAMA **283:**108–109.
5. **Cuzick, J., C. J. Meijer, and J. M. Walboomers.** 1998. Screening for cervical cancer. Lancet **351:**1439–1440.
6. **Gravitt, P. E., C. L. Peyton, T. Q. Alessi, C. M. Wheeler, F. Coutlee, A. Hildesheim, M. H. Schiffman, D. R. Scott, and R. J. Apple.** 2000. Improved amplification of genital human papillomaviruses. J. Clin. Microbiol. **38:**357–361.
7. **Husnjak, K., M. Grce, L. Magdic, and K. Pavelic.** 2000. Comparison of five different polymerase chain reaction methods for detection of human papillomavirus in cervical cell specimens. J. Virol. Methods **88:**125–134.
8. **Jacobs, M. V., P. J. F. Snijders, A. J. C. van den Brule, T. J. M. Helmerhorst, C. J. L. M. Meijer, and J. M. M. Walboomers.** 1997. A general primer GP5+/GP6+-mediated PCR-enzyme immunoassay method for rapid detection of 14 high-risk and 6 low-risk human papillomavirus genotypes in cervical scrapings. J. Clin. Microbiol. **35:**791–795.
9. **Jacobs, M. V., P. J. Snijders, F. J. Voorhorst, J. Dillner, O. Forslund, B. Johansson, M. von Knebel Doeberitz, C. J. Meijer, T. Meyer, I. Nindl, H. Pfister, E. Stockfleth, A. Strand, G. Wadell, and J. M. Walboomers.** 1999. Reliable high risk HPV DNA testing by polymerase chain reaction: an intermethod and intramethod comparison. J. Clin. Pathol. **52:**498–503.
10. **Josefsson, A. M., P. K. Magnusson, N. Ylitalo, P. Sorensen, P. Qwarforth-Tubbin, P. K. Andersen, M. Melbye, H. O. Adami, and U. B. Gyllensten.** 2000. Viral load of human papilloma virus 16 as a determinant for development of cervical carcinoma in situ: a nested case-control study. Lancet **355:** 2189–2193.
11. **Lampertico, P., J. S. Malter, M. Colombo, and M. A. Gerber.** 1990. Detection of hepatitis B virus DNA in formalin-fixed, paraffin-embedded liver tissue by the polymerase chain reaction. Am. J. Pathol. **137:**253–258.
12. **Meijer, C. J., T. J. Helmerhorst, L. Rozendaal, J. C. van der Linden, F. J. Voorhorst, and J. M. Walboomers.** 1998. HPV typing and testing in gynaecological pathology: has the time come? Histopathology **33:**83–86.
13. **Meissner, J. D.** 1999. Nucleotide sequences and further characterization of human papillomavirus DNA present in the CaSki, SiHa and HeLa cervical carcinoma cell lines. J. Gen. Virol. **80:**1725–1733.
14. **Nelson, J. H., G. A. Hawkins, K. Edlund, M. Evander, L. Kjellberg, G. Wadell, J. Dillner, T. Gerasimova, A. L. Coker, L. Pirisi, D. Petereit, and P. F. Lambert.** 2000. A novel and rapid PCR-based method for genotyping human papillomaviruses in clinical samples. J. Clin. Microbiol. **38:**688–695.
15. **Nindl, I., M. Jacobs, J. M. Walboomers, C. J. Meijer, H. Pfister, U. Wieland, T. Meyer, E. Stockfleth, R. Klaes, M. von Knebel Doeberitz, A. Schneider, and M. Duerst.** 1999. Interlaboratory agreement of different human papillomavirus DNA detection and typing assays in cervical scrapes. Int. J. Cancer **81:**666–668.
16. **Parkin, D. M., E. Laara, and C. S. Muir.** 1988. Estimates of the worldwide frequency of sixteen major cancers in 1980. Int. J. Cancer **41:**184–197.
17. **Pfister, H.** 1996. The role of human papillomavirus in anogenital cancer. Obstet. Gynecol. Clin. North Am. **23:**579–595.
18. **Sasieni, P., J. Cuzick, and E. Farmery.** 1995. Accelerated decline in cervical cancer mortality in England and Wales. Lancet **346:**1566–1567.
19. **Swan, D. C., R. A. Tucker, G. Tortolero-Luna, M. F. Mitchell, L. Wideroff, E. R. Unger, R. A. Nisenbaum, W. C. Reeves, and J. P. Icenogle.** 1999. Human papillomavirus (HPV) DNA copy number is dependent on grade of cervical disease and HPV type. J. Clin. Microbiol. **37:**1030–1034.
20. **Thelwell, N., S. Millington, A. Solinas, J. Booth, and T. Brown.** 2000. Mode of action and application of scorpion primers to mutation detection. Nucleic Acids Res. **28:**3752–3761.
21. **Vernon, S. D., E. R. Unger, and D. Williams.** 2000. Comparison of human papillomavirus detection and typing by cycle sequencing, line blotting, and hybrid capture. J. Clin. Microbiol. **38:**651–655.
22. **Walboomers, J. M., M. V. Jacobs, M. M. Manos, F. X. Bosch, J. A. Kummer, K. V. Shah, P. J. Snijders, J. Peto, C. J. Meijer, and N. Munoz.** 1999. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J. Pathol. **189:**12–19.
23. **Wheeler, C. M., T. Yamada, A. Hildesheim, and S. A. Jenison.** 1997. Human papillomavirus type 16 sequence variants: identification by E6 and L1 lineage-specific hybridization. J. Clin. Microbiol. **35:**11–19.
24. **Whitcombe, D., J. Theaker, S. P. Guy, T. Brown, and S. Little.** 1999. Detection of PCR products using self-probing amplicons and fluorescence. Nat. Biotechnol. **17:**804–807.
25. **Wise, J.** 2000. UK pilot scheme for HPV testing announced. BMJ **320:**600.
26. **Womack, S. D., Z. M. Chirenje, P. D. Blumenthal, L. Gaffikin, J. A. McGrath, T. Chipato, E. Ngwalle, and K. V. Shah.** 2000. Evaluation of a human papillomavirus assay in cervical screening in Zimbabwe. Br. J. Obstet. Gynaecol. **107:**33–39.
27. **Yamada, T., M. M. Manos, J. Peto, C. E. Greer, N. Munoz, F. X. Bosch, and C. M. Wheeler.** 1997. Human papillomavirus type 16 sequence variation in cervical cancers: a worldwide perspective. J. Virol. **71:**2463–2472.
28. **Ylitalo, N., P. Sorensen, A. M. Josefsson, P. K. Magnusson, P. K. Andersen, J. Ponten, H. O. Adami, U. B. Gyllensten, and M. Melbye.** 2000. Consistent high viral load of human papillomavirus 16 and risk of cervical carcinoma in situ: a nested case-control study. Lancet **355:**2194–2198.
29. **Zerbini, M., S. Venturoli, M. Cricca, G. Gallinella, P. De Simone, S. Costa, D. Santini, and M. Musiani.** 2001. Distribution and viral load of type specific HPVs in different cervical lesions as detected by PCR-ELISA. J. Clin. Pathol. **54:**377–380.