Semiautomated Detection of Human Papillomavirus DNA of High and Low Oncogenic Potential in Cervical Smears

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Detection of DNA from human papillomaviruses (HPV) of high and intermediate oncogenic risk in cervical smears may predict the presence of cervical cancer or may indicate precancerous changes. Here we describe a semiautomated polymerase chain reaction system for the detection and classification of HPV DNA that is present in clinically significant amounts in routine cervical scrapes.

Indexing Terms: polymerase chain reaction/cancer screening/cervical cancer

Cervical cancer is the second most common cancer in women worldwide. In most developed countries, the disease is partly controlled by cervical cytological surveillance; dyskaryosis in exfoliated cells is taken to indicate the presence of precancerous changes (cervical intraepithelial neoplasia, CIN) or cancer.5 In the UK, cervical smears from ~5 million women are examined annually. Severe or moderate dyskaryosis is detected in ~1.5% of these women, and 60–70% of these have histologically confirmed high-grade lesions (CIN grade 2 or 3). Mild or borderline dyskaryosis is detected in 5% of women, but only 20–30% have CIN 2/3. For an effective selective management policy for women with mild or borderline dyskaryosis, there is a need to distinguish those with high-grade lesions, who require early hospital referral, from the majority who may be more appropriately managed by cytological follow-up. The close association we have found between large amounts of DNA from human papillomavirus (HPV) types of high or intermediate oncogenic risk and cervical cancers or high-grade lesions (1, 2) suggests that HPV DNA might be used as an additional marker for major cervical disease.

Both Southern transfers and in situ hybridization have been used to detect HPV DNA, but neither of these is suitable for mass screening. More recently, the Hybrid Capture Assay (Digene Diagnostics, Silver Spring, MD) has been successfully used in a number of laboratories. However, a substantial proportion of the smears we received fell short of the minimum requirement of 1 µg of total DNA for a satisfactory test. We therefore explored the polymerase chain reaction (PCR) as a practicable alternative. We used a protocol with deliberately reduced sensitivity to avoid problems with contamination and inhibitors (1). In a pilot study involving 105 women referred to a colposcopy clinic because of smears with repeated mild or borderline results, we used type-specific PCR to identify HPV16, 18, 31, and 33 DNA in cervical scrapes, and accurately predicted 14 of the 22 women who showed histological evidence of high-grade disease (3). Type-specific PCR is labor intensive, and confirmation of the identity of type-specific PCR products by gel electrophoresis is time consuming. The technique would have only limited value in a cytological screening program where large numbers of routine cervical samples have to be examined at a reasonable cost. We show here that a BIOMEK 1000 laboratory workstation (Beckman Instruments, High Wycombe, UK) could be used to process a standardized commercial system, the SHARP (Solution Hybridization Assay for PCR Products) Signal™ (Digene Diagnostics), for the semiautomated detection of HPV DNA of both high- and low-risk types.

Materials and Methods

A BIOMEK 1000 was kindly loaned to the Department of Mathematics, Statistics and Epidemiology, Imperial Cancer Research Fund UK (ICRF) by Beckman Instruments (UK). The BIOMEK is a programmable liquid-handling instrument with motorized tool holder and work platform. The platform has four positions that can accommodate a (microtiter format) pipette tip box, plates, reagent reservoirs or tube racks (three), and places for up to four pipetting or measuring tools. Automatic plate washing facilities are also provided via a peristaltic pump and vacuum line. The model we used was fitted with an optional spectrophotometry accessory. Complex operations could only be semiautomated because of the limited number of positions on the platform, but a complete interfacing workstation with robotic arm (BIOMEK SL High Capacity System) is also available that allows for full automation if required. PCR and probe hybridizations were carried out on an Omnigene thermal cycler (Hybaid, Teddington, Middlesex, UK) with the use of polycarbonate microtiter trays and lids (Omniplates).

Type-specific PCR was carried out manually for HPV types 16, 18, 31, and 33. Gel electrophoresis was used to detect and quantify the amplified products as described previously (2), except that microtiter trays were used for the PCR in place of tubes and the cycling was adjusted to take account of the resulting increased lag times. For the semiautomated procedure we used manual methods to prepare the master PCR mix and the
initial dilutions of expensive detection reagents, and to wash the detection plates. This was because our scale of operation did not warrant full automation at this stage. All subsequent operations preceding and following the PCR itself were handled by the BIOMEK. The BIOMEK is programmed so that pipetting and measuring functions, built into the software, are assembled into flexible subroutines and methods designed to emulate manual procedures. All reagents and samples were dispensed and stored in Beckman deep-well microtiter plates, and all reagent transfers were achieved with Beckman pipetting tools and sterile tips.

The SHARP Signal system, HPV consensus primers, and type-specific probes and standards were kindly provided to the ICRF by A. Lorincz and J. Lazar (Diogene Diagnostics), who also advised us on the experimental procedures. The detection system is based on liquid-phase hybridization of an RNA probe to a biotinylated PCR product, capture of the RNA-DNA hybrids onto streptavidin-coated plates, and subsequent detection and quantification of captured hybrids with an alkaline phosphatase-labeled antibody specific for RNA-DNA hybrids. For the semiautomated procedure we used consensus PCR primers (4), one of which was biotinylated, to amplify a region of the HPV late protein 1 gene and two pools of type-specific probes for detecting low-risk (probe mix A: types 6, 11, 42, 45, 44) or high-risk (probe mix B: types 16, 18, 31, 33, 35, 46, 51, 52, 56) HPV types. All operations except plate-washing (as discussed above) and incubations were carried out on the BIOMEK.

All subjects were treated in accordance with the standards of University College London Medical School ethical committee. Cervical scrapes (n = 108) from the Margaret Pyke Center, London, UK, were collected in sterile phosphate-buffered saline. Half of each specimen was extracted and examined for HPV16, 18, 31, and 33 by using type-specific primers as previously described (2). The distribution of HPV DNAs in the specimens was as shown in Table 1. The remainder of each specimen was simply boiled for 10 min and tested by the semiautomated system described below.

The PCR was carried out on 4-μL samples with the use of HPV consensus primers (MY09 and MY11-biotinylated) (4) in a final volume of 50 μL overlaid with oil. The program was as follows: (a) denaturation at 97°C for 30 s; (b) enzyme addition; hold at 85°C [10 μL of AmpliTaq polymerase (Cetus, Emeryville, CA) 15 kU/L, was added manually by using a multichannel pipette]; (c) 40 cycles of: 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min; and (d) final extension at 72°C for 8 min. The times given represent times at the given temperatures that were determined from prior measurements with an in-well thermocouple. All steps in the detection of the HPV PCR products (including denaturation, hybridization, capture, and enzymatic detection) were carried out according to the manufacturers' protocols but with use of the BIOMEK.

Results and Discussion

The relation between increased HPV viral load and cytological abnormalities or risk of cervical disease was reported previously (5, 6). Our type-specific PCR results further suggest that the estimation of HPV DNA in cervical smears against known standards can correlate with clinical disease (1, 2). The approach we describe, however, can be used for routine purposes only if it can be automated. Such automation might take the form of multiplexed PCR with the use of type-specific primers or, alternatively, the use of consensus primers to reduce the number of individual PCR reactions required for each specimen. In both cases the relation between the amount of PCR product obtained and the amount of HPV DNA present in the specimen is expected to be weak because of varying degrees of competition between independently amplified templates or because of the differences in the efficiency of amplification of different HPV types when consensus primers are used. In this investigation we attempted to determine whether useful information can be obtained from an automated consensus protocol compared with the established manual, type-specific procedure.

The results for the 108 cervical scrapes are presented in Fig. 1. The amounts of signal obtained by using the high-risk probe (HPV types 16, 18, 31, 33, 35, 45, 51, 52, 56) or low-risk probe (HPV types 6, 11, 42, 43, 44) with the BIOMEK/SHARP procedure are related to the specific detection of large or small amounts of HPV types 16, 18, 31, and 33 by type-specific PCR. Under the conditions we used, a cutoff value of 0.3 A at 405 nm was established for the BIOMEK/SHARP procedure, above which the detection of high-risk HPV types corresponded well with the amounts of HPV 16, 18, 31, and 33 DNA (equivalent to 0.1 copy/cell) that we have previously defined as indicative of major clinical disease (1, 2). Only specimens with absorbancies >0.3 were considered positive (with either probe mix). Relative to the type-specific PCR, the overall sensitivity and specificity of the high-risk probe used in the BIOMEK/SHARP system (without regard for the HPV type present) were 85% and 84%, respectively. Four specimens with large amounts of high-risk HPV DNA by type-specific PCR (4 of 67, 6%) were positive with the low-risk probe mix A in the SHARP system (one CIN 3, one CIN 2, and two low-grade lesions), but these specimens were also positive with probe B. Eight specimens (8 of 31, 26%) gave results considered to be of uncertain clinical significance

<table>
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<th>Table 1. HPV DNA in 108 cervical scrapes.</th>
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<td><strong>HPV type</strong></td>
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<tr>
<td>Large*</td>
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<td>Small*</td>
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* More than one type present in large amounts.

* Negative for all four tests tested.

c >0.1 copy/exfoliated cell (mean), indicative of high-grade lesions (CIN 2, 3, and cancerous) (2).

c <0.1 copy/cell, clinical significance not clear (2).

c Not indicative of cervical disease (2).
The specimens are grouped according to type-specific PCR positivity: (a) HPV16, (b) HPV18, (c) HPV31, (d) HPV33, (e) large amount of more than one type, and (f) negative for all types tested. Individual types are further subgrouped by the amount of HPV DNA detected: L, large amount; S, small amount. For each subgroup we plot the absorbancy values for the individual specimens obtained in the BIOMEK/SHARP system by using either the low-risk probe mix (A, types 6, 11, 42, 43, 44) or high-risk probe mix (B, 16, 18, 31, 33, 35, 45, 51, 52, 56).

In the diagnosis of some infectious agents by PCR, detection of a single copy of the DNA may have profound implications (e.g., human immunodeficiency virus, congenital rubella). In contrast, the copy number of HPV DNAs that we have found to broadly distinguish historically confirmed high-grade from low-grade cervical lesions equates to ~100,000 copies per smear. The PCR protocol we used is therefore one of intentionally low sensitivity, and contamination leading to erroneous results has not been a problem in our laboratory. Nevertheless, we do take the precaution of preparing PCR reagents in a clean environment in sets (one set is used for each PCR run) and all manipulations by the BIOMEK operate inside a safety cabinet. The consensus primers and the amplified region are also dedicated for use only in this project. To monitor for PCR inhibitors, we add to randomly selected samples plasmid DNA containing known HPV sequences at concentrations corresponding to small amounts of DNA amplified by type-specific PCR. At the high sample dilutions we use, inhibitors are not a problem.

The time it takes to dispense one reagent to one 96-well tray varies from 2 to 5 min. Although this time is well within the capability of a manual operator using a multichannel pipette, the robot has the important advantage of eliminating operator error, which can readily occur in any highly repetitive procedure. With minor adjustments to suit individual needs, the Digene system is simple to perform, but detection of the amplified DNA requires a total incubation time of at least 4–5 h. The BIOMEK robotic arm would therefore be obligatory if a fully automated system were required.

Cytological surveillance has proved to be an effective measure in reducing the incidence of cervical cancer and precancer in recent years. However, it is evident that some women who present with mild cytological abnormalities have high-grade lesions, and that these women, even with an ideal cytological surveillance program, can be lost to follow-up. There is, therefore, a need for more sensitive and specific diagnostic tests that can detect tumor formation at an early stage, and the test for high-risk HPV DNA is an obvious candidate. We have shown that a simple, fully standardized PCR method based on the use of commercially available components produces objective data that correlate with clinical disease. The test can be carried out with little input labor and minimal technical expertise by a robotic system operating from preprogrammed subroutines. From data from our current semiautomated protocol of 184 specimens per run, we estimate that the unit cost of the BIOMEK/SHARP system would be comparable to cervical cytology (manuscript in preparation). This cost would be significantly reduced by full automation and increased throughput and should not represent a major financial burden when used as an adjunct to cytology in the screening program. Since the SHARP signal system is adaptable to identification of any PCR product for which a specific probe is available, the diagnostic approach reported here could be further exploited for automated identification of any normal or mutated nucleic acid cancer markers in other cancer screening programs.

References


