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Detection of human papillomavirus from liquid-based cytology specimens by in-house PCR: a pilot study

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Introduction

Cervical cancer is one of the three major malignancies in women worldwide¹ with half a million new cases diagnosed each year. In the UK alone there are, on average, 3200 new cases a year, resulting in approximately 1200 deaths.

Cervical cancer is the eleventh most common cause of cancer deaths in women in the UK, representing approximately 2% of all female cancers. There is strong epidemiological evidence to show that acquisition of high-risk human papillomavirus (HPV) phenotypes (i.e., HPV 16, 18, 31 and 33) is important in the development of cervical cancer,² and this group has been found in no less than 75% of cervical tumours.³

As a result of the mass-screening programme in place in the UK, a substantial decrease in the incidence of cancer occurred between 1988 and 1997, representing a fall of 42%. Whereas there were 10.4 cases per 100,000 in 1995, by 1999 the prevalence had dropped to 9.3 per 100,000.

A significant drop in the number of cancer cases has been achieved by the implementation of Papanicolaou (Pap) smear screening, which aims to detect a series of precancerous cytological changes termed cervical intraepithelial neoplasia (CIN).^{4,5} Identifying cervical cytology changes in conventional Pap smears involves taking a sample of cells from the transformation zone of the cervix, using a spatula or cytobrush. The cells collected are transferred to a microscope slide, fixed in alcohol, stained with a Papanicolaou technique, and then screened microscopically.

Identification of pathology in the transformation zone relies heavily on the skills of cytoscreeners, biomedical scientists and pathologists; however, the quality of the preparation can be compromised by the presence of inflammatory exudate, inadequate cellularity, or failure to sample the transformation zone adequately. In order to overcome some of these problems, liquid-based cytology (LBC) methodology has been developed, in which the patient's entire cervical cell sample is rinsed into a vial containing a preservative, and slides are then prepared for

ABSTRACT

The Papanicolaou smear remains the most common method for the detection of precancerous changes in cervical cytology. However, the introduction of a liquid-based cytology (LBC) technique expands the possibility of cervical intraepithelial neoplasia (CIN) diagnosis, and permits detection of precancerous changes and human papillomavirus (HPV) simultaneously. In the pilot study reported here, using an in-house polymerase chain reaction (PCR) method, high-grade HPV was detected in 32% of a cohort of 38 patients. This conventional PCR method could be developed for use on a real-time PCR platform or in a microtitre-well format and subsequently automated.

KEY WORDS: Cervical intraepithelial neoplasia. Cervical neoplasia. Human papillomavirus. Liquid-based cytology. Polymerase chain reaction

examination from the fluid sample. The advantages of LBC include more uniform specimen cellularity and the fact that there are fewer fields to examine.

The advantage of the polymerase chain reaction (PCR) as a diagnostic tool is its sensitivity, making it an ideal means of detecting low copy numbers of HPV DNA. Care and attention is required to avoid contamination of individual samples during collection and subsequent processing. In theory, 30–40 cycles can produce a million-fold amplification and the sensitivity is such that a single copy of HPV DNA can be detected. Samples of DNA isolated from Pap smears and paraffin wax-embedded specimens may be used as a template.

Two sets of primers (GP5+/GP6+⁶ and MY09 and MY11⁷) have been used extensively for HPV detection. Numerous HPV types have been implicated in cervical cancer, although HPV types 16, 18, 31 and 33 are most common. Thus, to be able to detect a broad spectrum of HPV types and yet be able to distinguish individual serotypes, Ting and Manos⁷ devised a pair of consensus primers that shared interspersed regions of DNA sequence homology, especially within the open reading frames (ORF) of the *E1* and *L1* genes. These workers identified regions of homology 20–25 bp in length, from which consensus primers that would amplify DNA from more than 25 types of genital HPV were designed.

The primers (MY11 for the positive strand and MY09 for the negative strand) are degenerate in several positions, which allows the possibility that more than one nucleotide

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Table 1. Position of first bases and size of HPV.

| HPV type | First base position | | Product size of L1 gene (bp) |
|----------|---------------------|-------------|------------------------------|
| | MY11 primer | MY09 primer | |
| HPV06 | 6722 | 7170 | 448 |
| HPV11 | 6707 | 7155 | 448 |
| HPV16 | 6584 | 7035 | 451 |
| HPV18 | 6558 | 7012 | 454 |
| HPV33 | 6539 | 6987 | 448 |

Table 2. PCR conditions for HPV DNA amplification.

| Ingredients | Volume (μ L) |
|---------------------|-------------------|
| Primer MY09 (x10) | 1 |
| Primer MY11 (x10) | 1 |
| Template DNA | 1.5 |
| PCR Master Mix | 12.5 |
| Nuclease free water | 9 |
| Total volume | 25 |

Table 3. Thermal cycling conditions for MY09/11 primers.

| Step | Temperature ($^{\circ}$ C) | Time (min) |
|----------------------|-----------------------------|------------|
| Initial denaturation | 94 | 5 |
| Denaturing | 94 | 1 |
| Annealing | 55 | 1 |
| Extension | 72 | 1 |
| Final extension | 72 | 5 |

can be inserted at a specific time. The size of the product obtained allows determination of the HPV type as shown in Table 1.

As the composition of the LBC medium in which cervical cells are preserved is unknown, the present study aims to determine the usefulness of this method in the diagnosis of HPV by an in-house PCR amplification method using the MY09/MY11 primers described above. Furthermore, characterisation of products is performed through DNA sequencing of the amplicons generated.

Materials and methods

A total of 38 LBC samples were collected randomly from apparently healthy patients (age range: 18–56; mean: 35 years) undergoing routine cervical cytology. Oligonucleotide primers for the detection of HPV types were synthesised and used according to the manufacturer's instructions (Invitrogen). The final concentration of primers used in each reaction was 0.5 μ mol/L.

The sequence of the primers were as follows: MY 11 (positive strand primer) 5' GCM CAG GGW CAT AAY AAT GG 3'; MY09 (negative strand primer) 5' CGT CCM ARR GGA WAC TGA TC, where M= A + C, R = A + G, W = A + T, Y = C + T.

DNA was extracted by an in-house method. Briefly, 1 mL well-mixed LBC fluid from each patient was transferred into 1.5 mL Eppendorf tubes using sterile disposable pipettes. The tubes were capped and centrifuged at 3500 rpm for 10 min. The supernatant was removed and 1 mL lysis buffer (10 mmol/L Tris [pH 7.4] containing 1 mmol/L EDTA, 1% SDS and 20 μ L 10 mg/mL proteinase K) was added to the deposit containing the cell pellet. The mixture was homogenised by vortex-mixing and incubated at 65 $^{\circ}$ C for 1 h.

The buffer containing proteinase K lyses cervical cells, which allowed both human and HPV DNA to be extracted. The tubes were then heated at 95 $^{\circ}$ C for 1 min to inactivate the proteinase K, and then cooled. The lysed material was divided into two Eppendorf tubes, one of which was kept at –70 $^{\circ}$ C for future use. To each of the remaining tubes, 1 mL phenol:chloroform:isoamyl alcohol (25:24:1 [v/v]; Sigma) was added, the contents were mixed and centrifuged at 3000 rpm for 10 min. The top layer was carefully removed and transferred to a new tube and an equal amount of chloroform was added, mixed and spun at 3000 rpm for 10 min.

The top layer was removed carefully into a clean tube and two volumes of cold 95% ethanol and 5 μ L 3 mol/L sodium acetate buffer were added, mixed well and kept in the freezer at –20 $^{\circ}$ C for 1 h. This last step permits the precipitation of DNA. The tube was then spun at 10,000 rpm for 10 min to pellet the extracted DNA and the supernatant was removed and discarded.

Then, 100 μ L 70% ethanol was added to the pellet (without mixing) and the tube spun at 10,000 rpm for 5 min. The supernatant was removed and discarded. 100 μ L of 95% ethanol was added to the pellet (without mixing) and centrifuged at 10,000 rpm for 10 min. The supernatant was removed and discarded. The pellet was allowed to air dry in a sterile incubator, following which the DNA pellet was resuspended carefully in 50 μ L sterile distilled water.

HPV DNA amplification was carried out in a Perkin Elmer 480 DNA thermal cycler (Perkin Elmer, Baltimore, USA) and PCR master mix (Promega, Southampton, UK) was used in 25 μ L reactions (Table 2). The constituents were added to a 0.5 mL Eppendorf tube, mixed briefly by vortex-mixing and then centrifuged at 2500 rpm for 30 sec. The reaction mix was then overlaid with 20 μ L sterile mineral oil, capped, and transferred to the thermal cycler for amplification. Thirty-five cycles of PCR were carried out using the cycling conditions described in Table 3.

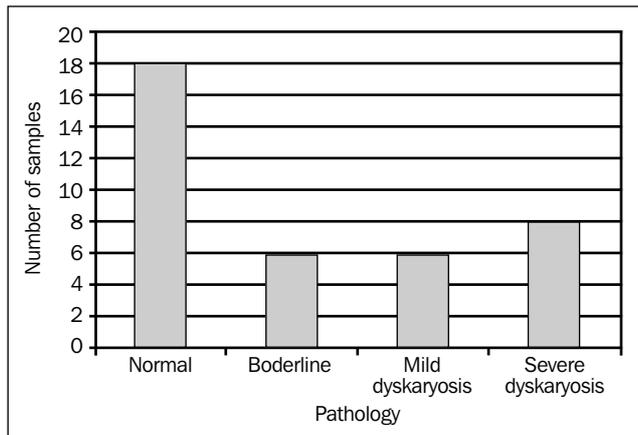
A negative control consisting of water instead of template DNA and a positive control consisting of DNA extracted from HeLa 229 cells infected with HPV 18 were also included.

Using a micropipette, 10 μ L PCR amplicon was removed carefully from the 0.5 mL Eppendorf tube into a fresh tube containing 2 μ L 6x loading dye (Promega). The mixture was loaded into the wells of a pre-cast 1% agarose gel in 1x TBE (89 mmol/L Tris, 89 mmol/L boric acid, 2 mmol/L EDTA [pH 8.3]) submerged in a Bio-Rad electrophoresis tank containing 1x TBE buffer. Also, 10 μ L 100 bp DNA ladder mixed with 2 μ L loading dye was included for size comparison. A voltage of 100 V was applied for 1 h to permit electrophoretic migration.

The gel was removed and stained in 100 mL 1x TBE buffer to which 10 μ L (10 mg/mL) ethidium bromide was added. The gel was allowed to stain for 10 min and then washed

Table 4. Age distribution of patients.

| Age group (years) | 18-22 | 23-27 | 28-32 | 33-37 | 38-42 | 43-47 | 48-52 | 53-57 |
|-------------------|-------|-------|-------|-------|-------|-------|-------|-------|
| Patient numbers | 4 | 5 | 6 | 11 | 7 | 1 | 1 | 3 |

Fig. 1. Cervical pathology of the cohort.

in distilled water. Ethidium bromide intercalates with the DNA bases, which then fluoresce under ultraviolet (UV) light from a transilluminator.

Results

Table 4 illustrates the age distribution of the patients tested.

Cytopathology

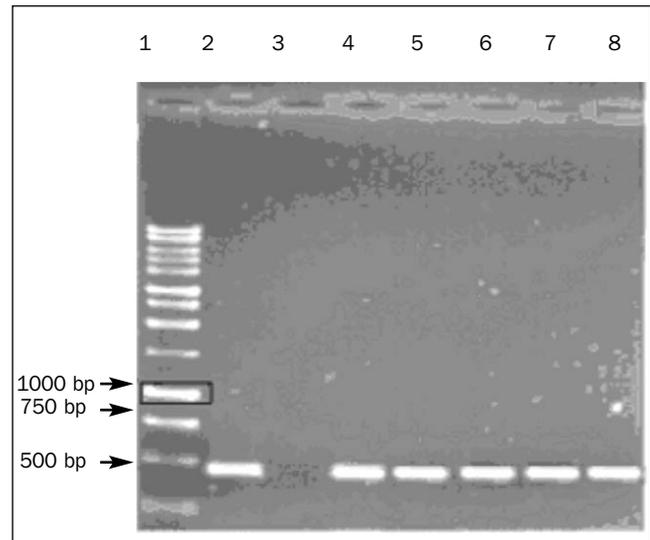
The results of cytopathological examination were presented as four outcomes: normal (no dyskaryosis), borderline changes, mild dyskaryosis, moderate-severe dyskaryosis. The distribution is illustrated in Figure 1. Among the cohort 47% were normal, 16% showed borderline changes requiring a further smear in six months, 16% showed mild dyskaryosis and 21% showed moderate to severe dyskaryosis (CIN 3).

Molecular detection of HPV

High-grade HPV was detected in 12 out of 38 samples (32%). The 33-37 age group produced the highest number of positive samples. Examples of the positive amplicons are shown in Figure 2.

Discussion

In the present study, all the patients who had moderate or severe dyskaryosis on cytology were also positive for high-grade HPV by the in-house PCR method used. However, two cases of mild dyskaryosis did not give a positive result with PCR. This suggests that either they were HPV-negative or that the virus was present but not detected (i.e., they were false-negatives) because HPV load was below the level of detection for the method. This could be verified by increasing the amount of target DNA in the amplification or increasing the number of amplification cycles to 40.

Fig. 2. Separation of amplicons on 1% agarose gel. Lane 1: 100 bp DNA (1000 bp standard band indicated); lane 2: positive control; lane 3: negative control; lanes 4-8: amplicons from patient samples. The amplicon sizes are slightly less than 500 bp, corresponding to that anticipated when using the MY09/MY11 degenerate primers.

In contrast, failure to detect HPV could be attributed to the fact that the set of consensus primers used did not detect HPV 31, which is also associated with cervical cancer, and could have been the cause of the dyskaryosis in these two cases. DNA degradation is another possibility that should be investigated by using a set of primers that target a housekeeping gene (e.g., β -actin) in human chromosomal DNA. However, this would only determine whether or not integrated viral genes were intact, as it would yield no information about unintegrated virus.

Clearly, samples should be analysed with a different set of HPV-specific primers but further negative results could be interpreted in three ways: either viral DNA was not present or extraction was inadequate or the product was degraded. The last possibility is unlikely because all the samples were collected and stored under similar conditions and those that were amplifiable gave a good yield of amplicons.

Overall detection rate of high-grade HPV in dyskaryosis was 86%. This correlates well with the results of other studies and implies that dyskaryosis in 10% of patients is not the result of viral infection. Thus, the two negative cases described may represent patients in whom dyskaryosis was not a consequence of high-grade HPV infection.

The purpose of the present study was to evaluate the performance characteristics of LBC in detecting HPV, as studies of the ThinPrep system (Cytoc, Boxborough, MA, USA) have shown that it was possible to detect the presence of HPV DNA by PCR from patients undergoing routine cervical cytology examination. Although the composition of the medium in which the cells are preserved is unclear,

nothing present appeared to interfere with the in-house PCR technique used in the present study. However, in order to remove potential inhibitors, it is advisable to separate the cervical cells from the medium by centrifugation, discard the medium and then wash the cells in isotonic saline before proceeding to DNA extraction.

Material isolated from LBC has also been tested in hybrid capture assays. A study by de Cremoux *et al.*⁸ investigated the efficiency of the Hybrid Capture 2 (HC2; Digene, Gaithersburg, MD, USA) HPV assay for the detection of cervical neoplasia in LBC. All patients were screened by both conventional Pap smear and ThinPrep LBC (Cytyc). HPV DNA was detected in the residual liquid-based material; however, their results included 111 false-positives (34 cross-reactions [1.90%] and 77 true false-positive [4.31%]), which were attributed to a contiguous strong chemiluminescence signal. Although they concluded that the HC2 assay was a reliable and sensitive test for HPV DNA detection, they did not advocate its use for large-scale screening for cervical neoplasia.

The in-house method used in the present study did not produce any false-positive reactions, is suitable for modification to a microtitre plate format (substituting an automated capillary detection system for the agarose gel), and could be adapted for real-time PCR platforms to determine viral load.

There are numerous advantages to the use of LBC. In addition to detecting precancerous changes in the cells in solution, adequate material remains on which to test for the presence of HPV. Although changes in the transformation zones are caused mainly by HPV, it has been suggested that other organisms, such as *Chlamydia trachomatis*^{9,10} and *Trichomonas vaginalis*,¹¹⁻¹⁴ may also be involved.

Currently, this group is studying the feasibility of detecting *C. trachomatis* and *T. vaginalis* from LBC samples by in-house PCR. □

Conclusions

Following National Institute for Clinical Excellence approval of the use of LBC in the NHS Cervical Screening Programme,¹⁵ the results of the pilot study presented here show that DNA extracted from the LBC medium can be used in molecular diagnostics. HPV DNA was detected in 32% of the cohort undergoing routine Pap screening and in 86% of those with dyskaryosis. However, investigation of a larger cohort is needed in order to fully appreciate the value of the in-house PCR used, but it is anticipated that a fully developed and tested in-house PCR would lower the cost of HPV testing considerably.

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