Use of bioinformatics and PCR in the search for ABC transporter homology among various bacteria.

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Use of bioinformatics and PCR in the search for ABC transporter homology among various bacteria

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ABSTRACT

Bioinformatics databases and search tools are utilised to produce polymerase chain reaction (PCR) primers for the amplification of an ABC transporter gene from the clinically important anaerobe *Finegoldia magna*. On sequencing, a 450 base pair amplicon showed homology with the amino acid transporter of *Enterococcus faecalis*. Little sequence data is available for *F. magna* and the newly isolated DNA could be a useful tool in the identification of this organism in clinical specimens.

KEY WORDS: ABC transporters.
Computational biology.
Finegoldia magna.

Introduction

In 1982, the first ABC transporter, the histidine permease *hisP*, was cloned and sequenced.[1] The ABC transporters (ABC Traffic ATPases[2]) now form part of one of the largest paralogous protein families known. They are highly conserved across species, they derive energy from the hydrolysis of ATP,[3] play a vital role in cell processes, and are characterised by their ability to translocate simple ions as well as complex proteins, even at low concentration, across the cell membrane.

The ABC transporters are used either for efflux or influx (some are involved in both) of solutes against a concentration gradient. However, they are substrate-specific and ABC transporters for amino acids, sugars, inorganic ions, polysaccharides, peptides and even proteins have been characterised. ABC transporters are involved in many basic cell functions such as signal transduction and protein secretion.[4]

A typical ABC transporter is made up of four parts: two membrane-associated domains and two ATP-binding domains. These subunits can be arranged as separate polypeptides or be fused in a variety of configurations. Both ATP-binding domains are required for normal function.

The transmembrane domains are highly hydrophobic[3] and consist of multiple α-helical segments that span the membrane. The ATP-binding domains are characterised in their primary structure by the presence of the Walker A and the Walker B motifs.[5] The Walker A sequence can be represented by GXXGXGKS/T, where 'X' can vary, and the Walker B sequence by hhhhD, where 'h' stands for hydrophobic. The Walker B sequence is preceded by a highly conserved sequence motif, LSGGQ/R/KQR. These conserved regions are shown in Fig. 1. The motif LSGGQ is considered to be a signature sequence for these proteins.[4]

Finegoldia magna (previously *Peptostreptococcus magnus*) is known to be a significant human pathogen,[6] its main habitat being human faeces, the urogenital tract and oral and skin epithelia. It is the most common Gram-positive anaerobic coccus isolated from clinical samples and is an opportunistic bacterium. It has been isolated from various sites of the body such as post-operative wounds, abscesses and ulcers[7]. Usually, *F. magna* is isolated from wounds where other bacteria are present; however, it has been isolated in pure culture in clinical samples, indicating that it has a higher pathogenicity than other species.[8]

In the diagnostic laboratory, this microorganism is classified as fastidious and is mostly overlooked in early cultures. Proper collection of specimens is important, as is its identification. Most strains of *F. magna* take more than 48 hours to grow and cultures for anaerobes take up to 48 hours in most routine microbiology laboratories. Thus, lack of a proper technique results in these anaerobic infections being diagnosed late or not at all.

The aim of this study is to amplify fragments of a gene encoding an ABC transporter in *F. magna* by searching databases for homology in this protein among various bacteria and other organisms. Furthermore, the development of a specific polymerase chain reaction (PCR) method to detect *F. magna*, based on the unconserved regions of the ABC transporter sequences, may be possible.

Materials and methods

The ATP-binding motif is the most highly conserved sequence in all the ABC transporters. GenBank and Swiss-Prot were interrogated for the ATP-binding ABC transporter genes in *Agrobacterium tumefaciens, Bacillus firmus, Encephalitozoon intestinalis, Escherichia coli, Haemophilus influenzae, Campylobacter jejuni, Bacillus subtilis, Staphylococcus aureus, Streptococcus pyogenes, Salmonella choleraesuis, Clostridium acetobutylicum* and also in *Arabidopsis thaliana*. These organisms were chosen on basis of their Gram-stain appearance and on the ATP-binding ABC transporter genes that have been cloned. Using the technique of conserved

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Fig. 1. Sequence alignment of the ATP-binding domains. The conserved sequences (Walker A and Walker B) are shown, as well as the highly conserved motif (signature sequence) that is unique to the members of the ABC transport superfamily (. low homology, : moderate homology, *complete identity).

Table 1. Two PCR products obtained using primers for Walker B.

Sequence 1: ***Finegoldia magna***
CGTCGAGAGGAGTTGCATTTGGGCCTGAAAATTTGGGAGTTCCAAGAGAAGA ACCCCTTNAGNGAGTAGATGAATGTCTTGAACTTGTGGGAATGANCNTTTANA AGNGACATTCACCAGCACCATTATCTGGAGGTCAAAAACAAAGAATTGCAATT GCTGGGATACTAGCAATGAATNCAAAGTGTTTGCTAATGGATGAGCCTACTA.
Sequence 2: ***Enterococcus faecalis***
AGAATATTGTTTTAGCACCAACCGATTTAGGTCGCTTGTCAAAAGACGAAGCCC CNTAAAAAAGCGTTAGAACTTTTAGAACGTGTTGGGTTGGCNCATAAAAAAGAT GCCTATCCAGATTCTTTATCTGGTGGTCAAAAACAACGTGTCGCAATTGCACGT GCGTTAGCGATGAATCCTGATATTATGCTTTTCGATGAGCCTACTAAGCCTACTA

sequence amplification (CSA) by PCR,[9] the genes encoding ATP-binding ABC transporters in various microorganisms were amplified.

Several sets of degenerate primers were designed according to the conserved areas of homology in the ATP-binding motif. The conserved motifs targeted for amplification for Walker A were GKST (forward) and DEAT (reverse), and for Walker B the conserved motifs were AMVFQ (forward) and DEPT (reverse). The primers for Walker A were forward 5′ GGHAARWSWACW 3′ and reverse 5′ WGTWGCYTCRTC 3′ and the primers for Walker B were forward 5′ GCWATGGTWTTYCAA 3′ and reverse 5′ WGTWGGYTCRTC 3′. The codon usage for *Micromonas micros* was used as that for *F. magna* was not available at the time the study was undertaken.

The correct combinations of primers used were based on the sequence alignment of *E. coli* published by Higgins in 1992. Only two sets of the degenerate primers produced amplicons. All the primers were tested on the DNA of a variety of aerobes and anaerobes which was extracted by the miniprep bacterial genomic DNA method.[10]

The PCR technique was carried out using standard Mastermix (Promega, 25 units/mL *Thermus aquaticus* [*Taq*] DNA polymerase, 200 μmol each of dATP, dGTP, dCTP and dTTP, and 1.5 mmol/L $MgCl_2$). The PCR reaction was carried out in a 25- μL volume and 1 μL DNA from each anaerobe and aerobe was used. The negative control included water instead of DNA. The PCR programme was run for 30 cycles and the annealing temperature used was 37°C.

American Type Cell Culture (ATCC) strains of *F. magna* (ATCC 29328) and *M. micros* (ATCC 33270) were used. Clinical isolates of organisms obtained from patients at University College London Hospital were also used and included (anaerobes) *F. magna, M. micros (Peptostreptococcus micros), Peptinophilus asaccharolyticus (Peptostreptococus assacharolyticus), Anaerococcus prevotii (Peptostreptococcus prevotii), Peptostreptococcus anaerobius, Prevotella melaninogenica, Prevotella bivia* and *Bacteroides fragilis*; and (aerobes) *E. coli, Pseudomonas aeruginosa, Enterococcus faecalis, Staphylococcus aureus, Staphylococcus epidermidis*, coagulase-negative staphylococci and group B streptococci.

The PCR amplicons were separated on 1% agarose gel in 1x Tris-boric acid-EDTA (TBE buffer; 0.45 mol/L Tris, 0.44 mol/L boric acid, 0.5 mol/L EDTA [pH 8.3]) by electrophoresis for 1 h at 100 V. In addition, some were sent for further sequencing (Cytomix, Cambridge, UK).

Results

The primers successfully amplified products from the DNA extracted from both aerobes and anaerobes. The PCR products that were obtained when using primers for Walker A were approximately 450 bp in size and are shown in Fig. 2 (anaerobes) and Fig. 3 (aerobes). The PCR products obtained when using primers for Walker B were approximately 360 bp in size and are shown in Fig. 4 (anaerobes) and Fig. 5 (aerobes). As more than one product was amplified, only those reactions that generated a single PCR product were sent for sequencing (Cytomix, Cambridge, UK).

The two PCR products obtained when using primers for Walker B were sequenced successfully and are shown in

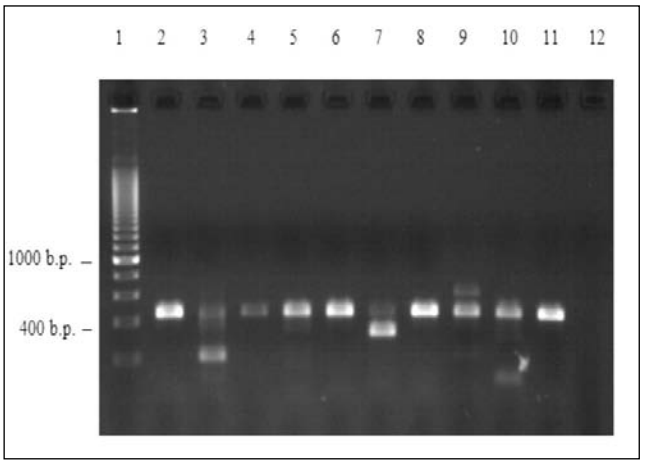

Fig. 2. PCR products of the anaerobes using Walker A primers. Lane 1: 200 bp ladder, lane 2: *F. magna* ATCC, lane 3: *F. magna*, lane 4: *M. micros* ATCC, lane 5: *M. micros*, lane 6: *Peptinophilus asaccharolytica* , lane 7: *Prevotella bivia*, lane 8: *Peptostreptococcus anaerobius*, lane 9: *A. prevotii*, lane 10: *B. fragilis*, lane 11: *Prevotella melaninogenica*, lane 12: negative control.

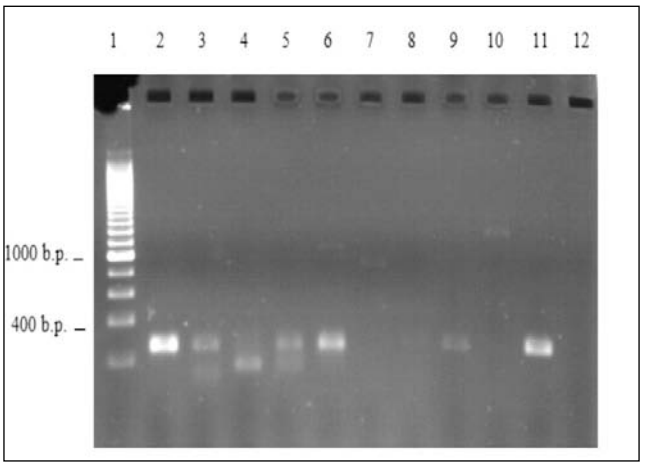

Fig. 4. PCR products of the anaerobes using Walker B primers. Lane 1200 bp ladder, lane 2: *F. magna* ATCC, lane 3: *F. magna*, lane 4: *M. micros* ATCC, lane 5: *M. micros*, lane 6: *Peptinophilus asaccharolyticus*, lane 7: *Prevotella bivia*, lane 8: *Peptostreptococcus anaerobius*, lane 9: *A. prevotii*, lane 10: *B. fragilis*, lane 11: *Prevotella melaninogenica*, lane 12: negative control.

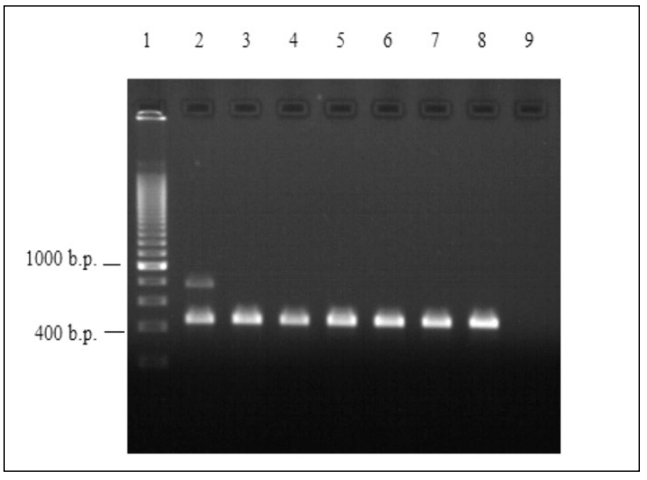

Fig. 3. PCR products of the aerobes using Walker A primers. Lane 1: 200 bp ladder, lane 2: *E. coli*, lane 3: group B streptococcus, lane 4: *S. aureus*, lane 5: *S. epidermidis*, lane 6: coagulase-negative staphylococcus, lane 7: *E. faecalis*, lane 8: *Pseudomonas aeruginosa*, lane 9: negative control.

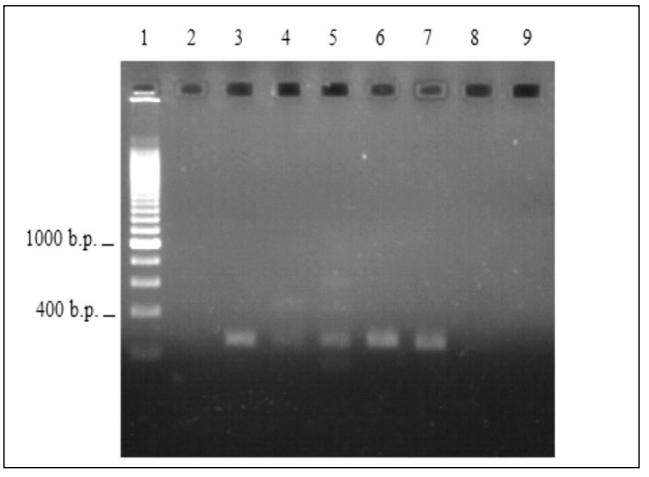

Fig. 5. PCR products of the aerobes using Walker B primers. Lane 1: 200 bp ladder, lane 2: *E. coli*, lane 3: group b streptococcus, lane 4: *S. aureus*, lane 5: *S. epidermidis*, lane 6: coagulase-negative staphylococcus, lane 7: *E. faecalis*, lane 8: *Pseudomonas aeruginosa*, lane 9: negative control.

Table 1. They were first translated into amino acid sequences and then analysed using the EMBOSS transeq software (www.ebi.ac.uk/emboss/transeq/). The results of the translations included the ABC transporter signature sequence LSGGQ (data not shown).

Discussion

In this study, the ABC transporter genes were amplified correctly using primer designs based on homology and conserved sequence amplification. Previously, this technique was used successfully in the cloning of the glycosidase genes from the protozoan *Tritrichomonas foetus*.[9]

Although the ABC transporter gene sequence in *F. magna* should be similar to ABC transporter genes in other microorganisms, it would be expected to show differences because the four domains of the ABC transporters are encoded by different polypeptides. Based on this difference in sequence, a specific PCR method to detect only the ABC transporter gene in *F. magna* can be designed and used as a diagnostic tool for the rapid identification of *F. magna* in clinical samples.

However, the short ATP-binding motifs (Walker motifs) are known to occur on other types of transporters (eg F-type ATPases, P-type ATPases and the Ars arsenite/arsenate export system).[11] Interestingly, when the sequences obtained in this study were translated into amino acid sequences, the consensus and signature sequence LSGGQ was present. This special sequence identifies members of the ABC superfamily.[11]

Data generated from this study are new, as no information on ABC transporters for *F. magna* is currently available in any bioinformatics database. Interestingly, the *F. magna* sequence data showed 63% homology with the ABC transporter in *Clostridium perfringens*. The latter is also an anaerobe, which

suggests that the sequence obtained is genomic information for *F. magna*. More importantly, the sequence data showed 59% homology with the ATPase component of the anaerobes *C. acetobutylicum* and *Thermoanaerobacter tengcongensis*. The *F. magna* sequence obtained showed 54% homology with the ABC transporter, more specifically the ATP-binding domain, of *Streptococcus pneumoniae* and *S. pyogenes*. This reflects the specificity of the primers used in this study.

The sequence obtained from the clinical isolate of *F. magna* showed 96% homology with the multidrug-resistance protein (cel:C54D1.1). Not surprisingly, this clinical isolate was obtained from a wound infection.

ABC transporters have been associated with antibiotic resistance, and multidrug resistance pumps have also been found in other pathogens.[12] ABC transporters actively pump antibiotics out of the cell, and some that are found in microorganisms of medical importance have been identified. One such example is the ABC protein MrsA, which has been postulated to cause erythromycin resistance in staphylococci.[13] However, not all antibiotic resistance by export involves ABC proteins.

The sequence data obtained for the amplicon from *Enterococcus faecalis* showed 90% homology with the *abc* gene coding for an amino acid ABC transporter in an *E. faecalis* vancomycin-resistant V583 clinical isolate. Thus, *E. faecalis* was identified to the species level and the amplicon obtained was most probably that of the amino acid ABC transporter.

Genome research that involves comparative analysis of ABC transporters in completed microbial genomes will provide greater insight into their roles and functions. One such study was carried out by Tomii and Kaneshisa.[14] They analysed various completed genomes and studied the number of ABC-type ATP-binding proteins that may be present in Gram-negative bacteria, Gram-positive bacteria, cyanobacteria and archaea. They also studied the functional predictions of the ATP-binding proteins. In this way, the different types and numbers of ABC transporters can be lodged in a database so that ABC transporters can be identified in other microorganisms using bioinformatics and homology.

Currently, antibiotics are prescribed prophylactically to the elderly when they undergo dental procedures because the transfer of *F. magna* infections to hip prostheses have occurred in such cases.[15] Use of antibiotics in this way will lead to increasing resistance among the different *F. magna* isolates.

Clearly, in the face of growing antibiotic resistance of microorganisms, the study of microbial ABC transporters is of the utmost importance. In this respect, the use of rapid DNA-based diagnostic tests[16] will prove extremely useful. □

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

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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

Introduction

Cervical cancer is one of the three major malignancies in women worldwide[1] 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 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 MY11[7]) 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 Manos[7] 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
	MY11 primer	MY09 primer	of *L1* gene (bp)
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 (μL)
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 (˚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 μmol/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 μL 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˚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˚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˚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 μL 3 mol/L sodium acetate buffer were added, mixed well and kept in the freezer at –20˚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 μL 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 μL 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 μL 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 μL 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 μL 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.

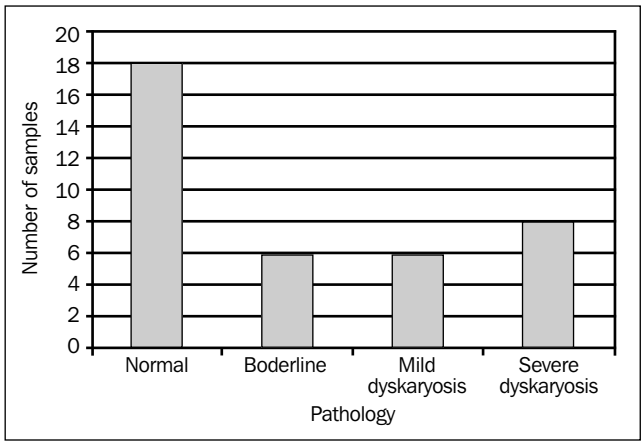
Using a micropipette, 10 μL PCR amplicon was removed carefully from the 0.5 mL Eppendorf tube into a fresh tube containing 2 μL 6x loading dye (Promega). The mixture was loaded into the wells of a pre-cast 1% agarose gel in 1x TBE (89 mmol/L Tris, 89 mmol/L boric acid, 2 mmol/L EDTA [pH 8.3]) submerged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

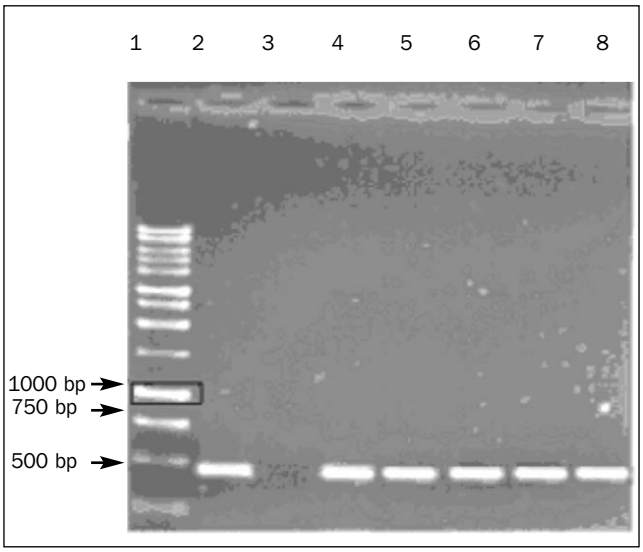
Molecular detection of HPV

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/MY11degenerate 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 (Cytyc, 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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