Comparative analysis of human papillomavirus detection by PCR and non-isotopic in situ hybridisation

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Abstract

Aims—To assess the relative diagnostic performance of the polymerase chain reaction (PCR) and non-isotopic in situ hybridisation (NISH) and to correlate these data with cytopathological assessment.

Methods—Paired analysis of human papillomavirus (HPV) detection was performed by PCR and NISH on exfoliated cervical cells from 122 women attending a routine gynaecological examination. PCR amplification followed by generic and HPV type specific hybridisation was compared with NISH on a parallel cervical smear.

Results—Overall, 32 cases were positive by NISH and 61 positive by PCR. Of the 105 cases in which both PCR and NISH were interpretable, 76 (26%) were normal smears, 20 of which were HPV positive by NISH and 37 (49%) by PCR. Of 17 borderline smears, two were NISH positive and 12 PCR positive. Eight of nine smears containing koilocytes were positive by NISH and seven by PCR. Of three dyskeratotic smears, none were NISH and two were PCR positive. The concordance of NISH and PCR in these samples was 57%. To assess sampling error, NISH and PCR were performed on an additional 50 cases using aliquots from the same sample. This increased the concordance between assays to 74%. Filter hybridisation of PCR products with the cocktail of probes used in NISH (under low and high stringency conditions) demonstrated that several cases of NISH positivity could be accounted for by cross-hybridisation to HPV types identified by PCR but not present in the NISH probe cocktail.

Conclusions—Sampling error and potential cross-hybridisation of probe and target should be considered in interpretation of these techniques. PCR is more sensitive because it provides for the amplification of target DNA sequences. In addition, the PCR assay utilised in this study detects a wider range of HPV types than are contained in the cocktails used for NISH. However, PCR assays detect viral DNA present both within cells and in cervical fluid whereas NISH permits morphological localisation.

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Human papillomavirus (HPV) infection (particularly with HPV 16, 18, 31, 33, 35, 45, 52, and 56) has been implicated as an early event in the natural history of cervical intraepithelial neoplasia (CIN) grades II and III and invasive squamous cell carcinoma.^{1,2} Colposcopic assessment and histopathological and cytopathological testing do not, however, detect non-productive viral infection, or the presence of integrated virus. This can only be demonstrated by nucleic acid hybridisation. The presence of HPV DNA in cytological samples has been demonstrated using a variety of molecular techniques, including filter hybridisation,^{3,4} in situ hybridisation (NISH),^{5,6} PCR⁷⁻⁹ and the recently described hybrid capture technique.¹⁰ In situ hybridisation offers the advantage of providing localisation of HPV genomes to epithelial cells. False positives due to laboratory or clinical contamination, a potential problem with PCR analysis,⁶ are therefore obviated. However, PCR has the advantage of high sensitivity and flexibility, being able to detect very low levels of virus in clinical samples^{8,9} but it does not discriminate between cellular infection and the presence of extracellular HPV which may represent an incidental, non-infective passenger virus. Moreover, the biological significance of the presence of low copy number HPV is unclear.

There are many studies demonstrating HPV DNA in exfoliated cervical material utilising NISH and PCR,^{5,6,11-15} but a direct comparison of these techniques has not been performed previously. The aims of this study were twofold: first, to assess the relative diagnostic performance of NISH and PCR and, second, to correlate these data with cytopathological assessment. To address these issues, parallel cervical samples were analysed from 122 women undergoing routine gynaecological examination. A direct comparison between NISH and PCR was made on aliquots of cells/fluid from the same cervical sample to determine the effects of sampling error and potential cross-hybridisation of probe and target DNAs in the NISH assay. The samples were tested for the presence of HPV by PCR and NISH in separate laboratories and the results evaluated for each cytological diagnostic group.

Methods

Women (n = 122) undergoing a routine gynaecological examination were recruited from two sites; Greenville, North Carolina and San

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Diego, California, USA between March and May 1990. A cervical swab was taken for PCR analysis and placed in viral transport medium (DiGene Diagnostics Inc., Silver Springs, Maryland, USA). The suspended cells recovered from the swab were assayed for the presence of HPV by PCR. A cervical smear was taken at the same time from each patient and fixed immediately in the standard way. NISH was performed on this smear which was then counterstained with haematoxylin and evaluated both cytopathologically and for the presence of NISH signal.^{6,16} Cytopathology, NISH (Oxford University, Roche Biomedical Laboratories) and PCR analysis (Cetus Corporation, Roche Biomedical Laboratories) were performed. Aliquots from the transport medium of an additional 50 cases were analysed by both NISH and PCR (see later).

POLYMERASE CHAIN REACTION

PCR analysis was performed as described by Bauer *et al.*⁹ Briefly, a 450 base pair fragment of the L1 gene was amplified using a set of degenerate consensus primers shown to detect at least 25 anogenital HPV types. PCR products were then analysed with both generic probes and type specific oligonucleotide probes and detected by enhanced chemiluminescence. A β -globin fragment (256 base pairs) was amplified in all cases to ensure adequate DNA quantity and quality. Probe specificity was determined using plasmid derived PCR products from HPV 6, 11, 16, 18, 31, 33, 35, 39, and 45.

FILTER HYBRIDISATION

Samples in transport medium were spotted on to nitrocellulose and hybridised with genomic HPV probes labelled by nick translation with ³²P (0.5–1 × 10⁸ dpm).¹⁷ The hybridisation buffer consisted of 50% formamide, 6 × standard saline citrate (SSC), 5 × Denhardt's solution, 0.5% sodium dodecyl sulphate (SDS) and 100 µg/ml salmon sperm DNA at 42°C (Tm – 20°C). Washes were carried out in either 2 × SSC at 40–60°C (Tm – 30 to 50°C) or 0.1 × SSC at 65°C (Tm – 7°C) and signal detected by autoradiography.

NISH ON CERVICAL SMEARS

In situ hybridisation was performed on routine cervical smears as described previously.⁶ Briefly, after fixation and nucleic acid unmasking with 1 µg/ml proteinase K, a cocktail of HPV 6, 11, 16, 18, 31, and 33 probes (2 ng/µl each) labelled with digoxigenin by nick translation¹⁸ was added to the hybridisation mix (50% (v/v) formamide, 5% (w/v) dextran sulphate, 2 × SSC (1 × SSC = 0.15 M sodium chloride, 0.015 M sodium citrate), 50 mM Tris-HCl, pH 7.2, 5 mM EDTA, 0.1% (w/v) sodium pyrophosphate, 0.2% (w/v) polyvinylpyrrolidone MW 400 000, 0.2% (w/v) Ficoll MW 400 000, and 200 ng/µl sheared human DNA). Target DNA and probes were denatured simultaneously at 95°C for 15 minutes and hybridised at 42°C for two hours.¹⁹

After hybridisation, smears were washed twice in 4 × SSC and then soaked in blocking agent (TBT) composed of 50 mM Tris-HCl (pH 7.2), 100 mM NaCl, 1 mM MgCl₂ (TBS) containing 3% (w/v) bovine serum albumin, 0.05% (v/v) Triton X-100. Bound probe was detected using a three step peroxidase based technique.²⁰ A red NISH signal was developed by incubation in 3-amino-9-ethylcarbazole with H₂O₂ (Zymed, California, USA) for 10 minutes. The reaction was stopped by washing in water. Smears were counterstained progressively with haematoxylin for 10–15 seconds and mounted in glycerol jelly.¹⁸ To analyse cross-hybridisation in NISH experiments, an additional 50 cases were investigated by spotting 50 µl of each sample in viral transport medium on to slides and processing the specimens as for routine cervical smears. An aliquot of each sample was also analysed by PCR (see above).

CONTROLS

In each PCR assay, SiHa DNA was used as a positive control and a "no DNA" reagent control was used as a negative control. Controls in all NISH experiments included CaSki cell smears²¹ and cytologically normal cervical smears (from a separate cohort) hybridised with HPV 16 and total human DNA, respectively.²² Multiple nuclear signals were obtained in CaSki cells²⁰ and total human DNA labelled every nucleus in control smears.

CYTOPATHOLOGICAL ASSESSMENT OF NISH SMEARS

After NISH, the smears were counterstained with haematoxylin, cytologically assessed and assigned to four diagnostic groups: smears regarded as being within normal limits; borderline smears where abnormalities were present but not diagnostic of either wart virus infection (WVI) or dyskaryosis; WVI where koilocytosis was present; and dyskaryotic smears.

Results

PCR ANALYSIS

Of the 122 cases, eight specimens were not included in the PCR analysis because of lack of amplification of the internal positive control (β -globin): three of these cases were positive by NISH. Sixty one of the remaining 114 (54%) cases were PCR positive after hybridisation with the generic HPV probe. Correlation of PCR positive samples with cytopathological diagnosis (table 1) demonstrated that 37 (of 76; 49%) smears were within normal limits, 12 (of 17) were borderline smears, seven (of nine) were smears showing wart virus changes and two (of three) were dyskaryotic smears. Thirty eight (64%) of the PCR positive specimens hybridised with the HPV generic probe but did not hybridise with the type specific probes. This reflects the presence of other HPV types such as HPV 42, 51, and 52, which were detected by the generic probe but did not hybridise with the type specific oligonucleotide probes.

Table 1 Cervical cytopathology and HPV detection by PCR and NISH

Cytopathology	n ₁	n ₂	NISH	PCR typed ^a	PCR all ^b
Normal	82	76	22	9	37*
Borderline	18	17	2	8	12*
WVI	10	9	8	3	7*
Mild/moderate dyskaryosis	3	3	0	1	2
Not determined	9	0	ND	2	3
Total	122	105	32	23	61

n₁ refers to the total number of cases in each diagnostic category and n₂ to those in which both NISH and PCR were interpretable.

* In six normal cases, one borderline and one WVI, PCR analysis was not performed as the samples were inadequate (see results).

ND = not determined due to excessive non-specific substrate deposition.

^a All cases typed by PCR as HPV 6, 11, 16, 18, 31, or 33.

^b All cases which hybridised with the generic HPV probes.

Figure 1 A cervical smear hybridised with a cocktail of nick-translated probes for HPV 6, 11, 16, 18, 31, and 33 and detected as described in Methods. Note the presence of nuclear signal in epithelial cells.

NISH ANALYSIS

In nine cases the NISH and cytological results were uninterpretable because of non-specific substrate deposition (three of these specimens were positive by PCR). Using a cocktail of nick-translated HPV probes (6, 11, 16, 18, 31, and 33) on the remaining 113 samples, NISH detected viral sequences in 32 (26%) (fig 1). HPV DNA was found in 22 of 82 smears within normal cytopathological limits, two of 18 borderline smears, eight of 10 smears showing diagnostic features of WVI and in none of three dyskaryotic smears.

COMPARISON OF NISH AND PCR ANALYSIS

Thirty two NISH and 61 PCR samples were positive for HPV DNA sequences. A direct comparison of PCR and NISH was possible for 105 cases (table 2). Twenty one cases were positive by both assays; 37 cases were PCR positive and NISH negative; eight were NISH

positive and PCR negative. Of the 76 cytologically normal smears analysed by both techniques, 20 (26%) were HPV positive by NISH and 37 (49%) by PCR. Of 17 borderline smears, two were NISH and 12 PCR positive. Seven of nine smears containing koilocytes were positive by NISH and the same seven of nine by PCR: there was one case in which PCR analysis was unsatisfactory and this sample was NISH positive. Of three cases with evidence of dyskaryosis, none were positive by NISH and two by PCR.

INVESTIGATION OF DISCORDANCE BETWEEN NISH AND PCR

There are three apparently discordant groups: NISH negative, PCR positive; NISH positive, PCR negative; and NISH positive, PCR generic probe positive—that is, either not typable or determined as HPV types not included in the probe cocktail used for NISH (that is, not HPV 6, 11, 16, 18, 31, or 33).

There are several possible explanations for discordance including sampling error, false positive NISH or PCR and false negative NISH or PCR. Furthermore, the presence of a NISH signal in cases shown by PCR to be infected by an HPV type other than those present in the NISH probe cocktail raises the possibility of probe cross-hybridisation in the NISH assay. To address the possibility of sampling error, NISH and PCR analysis of an additional 50 cases was carried out using separate aliquots from sample cell suspensions. Of these, 28 were NISH and 37 PCR positive. This gives a concordance of 74% (table 3) suggesting that sampling error was, at least in part, responsible for the original discordance.

A series of experiments was performed to test the hypothesis that the presence of a NISH signal in smears from patients whose samples were PCR positive for the generic probe only was because of cross-hybridisation between the nick-translated genomic HPV probe cocktail used in NISH and related HPV types in the sample. Repetition of NISH and PCR analysis in 50 cases as described above showed that 12 NISH positive cases were positive for HPV types other than HPV 6, 11, 16, 18, 31, and 33 (used in NISH experiments) by PCR. The remaining 13 concordant cases were typed specifically. The NISH experiments were repeated, incorporating a high stringency post-hybridisation washing step (50% formamide, 0.1 × SSC at 37°C; T_m + 4°C^{22,23}). Using this protocol, 15 of 25 NISH positive samples observed with low stringency washes were no longer positive. Six of the 10 remaining signals were from specimens identified as containing HPV 6, 11, 16, 18, 31, or 33—that is, the probes used for NISH hybridisation. A further set of filter hybridisation experiments was also performed using PCR products and a cocktail of nick-translated whole genomic probes for HPV 6, 11, 16, 18, and 31. At T_m – 20°C, hybridisation occurred between PCR products positive for only the generic probe and the nick-translated probe cocktail used for the NISH experiments. Signal was abolished in all cases

Table 2 Correlation of NISH and PCR analysis

NISH	PCR		
	Positive	Negative	Inadequate
Positive	21	8	3
Negative	37	39	5
Unsatisfactory	3	6	0

This gives an overall concordance of 49% and a concordance of 57% for samples in which both NISH and PCR results were available.

Table 3 Comparison of NISH and PCR performed on parallel aliquots of cells in suspension

NISH	PCR	
	Positive	Negative
Positive	25	3
Negative	8	6
Unsatisfactory	4	4

(with retention of signal from plasmid controls) at $T_m - 7^\circ\text{C}$ (fig 2). These two groups of experiments strongly support the hypothesis that cross-hybridisation of nick-translated genomic probes with other HPV types occurs under these conditions.

Figure 2 Hybridisation of L1 gene PCR products to nick-translated whole genomic probes. Plasmid derived PCR products from HPV 6, 11, 16, 18, and 31 were spotted in lanes X1–5, respectively, and products from 10 NISH positive, PCR “other” cases in lanes Y1–10. Lanes X6–10 contained no DNA. The filters were hybridised with a mixture of HPV 6, 11, 16, 18, and 31 probes and washed at stringencies of (A) $T_m - 50^\circ\text{C}$, (B) $T_m - 40^\circ\text{C}$, (C) $T_m - 30^\circ\text{C}$, and (D) $T_m - 7^\circ\text{C}$. Note the absence of signal from the test cases in (D).

Discussion

In situ hybridisation and PCR amplification are two frequently used techniques for the investigation of HPV infection in clinical material. Both techniques have advantages and disadvantages, with ISH being potentially limited by sensitivity and PCR by problems of product carry-over. The combined use of NISH and PCR amplification in cytological samples was therefore tested in this study. The use of a PCR system that amplifies DNA from a broad spectrum of HPV types with subsequent specific HPV typing by hybridisation permitted a detailed evaluation of these two techniques, given the proviso that only six probes were used in the NISH experiments. In normal smears more cases were considered positive by PCR than by NISH, with most of the PCR positive cases containing HPV types not present in the NISH probe cocktail. A significant number of NISH positive cases were also identified as containing HPV types not present in the nick-translated probe cocktail. This was also true for koilocytic smears, where only three of seven PCR positive samples contained HPV types present in the NISH probe cocktail but eight of 10 smears were NISH positive. These observations suggested that cross-hybridisation was occurring between nick-translated genomic HPV probes used for NISH and “other” HPV types (see below). The greater diagnostic sensitivity of PCR in borderline smears (12 of 18 were PCR positive compared with only two by NISH) may be because of either the presence of a small number of copies of HPV in cases showing only minor cytological changes (and hence reflect the greater absolute sensitivity of PCR), or the masking of epithelial cells by inflammatory cells.

The discordance between NISH and PCR, particularly in normal smears, is open to several interpretations. Firstly, sampling error may be responsible as the NISH and PCR analyses were performed on exfoliated cells obtained from different swabs. This was investigated by performing NISH and PCR on separate aliquots from the same specimen in 50 additional cases. A greater level of concordance was achieved (74%) and only three cases were NISH positive, PCR negative. Thus, NISH positive, PCR negative cases may be explained, at least in part, by sampling error. Possible explanations for NISH negative, PCR positive cases include sampling error, the broader spectrum of HPV types amplified or the greater sensitivity of PCR, particularly in situations where only low copy numbers of HPV are present. On the basis of the present data, each of these explanations, or a combination thereof, is possible for the predominance of PCR positive, NISH negative over PCR negative, NISH positive cases, both in the original series and in the cases in which separate aliquots from the same specimen were analysed.

The presence of NISH positive cases in which PCR showed that the infecting HPV type was one other than those present in the NISH probe cocktail suggests that the probes used in NISH cross-hybridise with other HPV types. The removal of signal using a high strin-

gency posthybridisation wash in the majority of such cases in the 50 additional specimens analysed supports the hypothesis that these findings are principally due to cross-hybridisation. The observation that signals due to other HPV types were not always removed whereas some due to probes present within the NISH probe cocktail were removed is, at first sight, surprising. However, it has been shown previously that the stringency of post-hybridisation washing is dependent on viral copy number as well as sequence.^{23,24} Signal may therefore be removed from a cell infected with a low copy number of a type present in the probe cocktail but not from a cell infected with a high copy number of a related virus. Additionally, this hypothesis was confirmed by the demonstration of cross-hybridisation between nick-translated genomic HPV probes with HPV targets on filters at a stringency of $T_m - 20^\circ\text{C}$. Signals were abolished by washing to $T_m - 7^\circ\text{C}$ indicating only partial homology between probe and target. It is clear therefore that NISH analysis may detect HPV types other than those included in the probe cocktail, a property which must be recognised in the interpretation of this technique.

In conclusion, NISH is capable of demonstrating cellular infection in conjunction with morphological analysis. PCR is of value in the assessment of unknown or less well characterised viral types but may also amplify contaminating exogenous and extracellular endogenous viral DNA, a particular problem where PCR product contamination occurs. NISH analysis does, however, detect a wider spectrum of HPV types than those included in the probe cocktail and permits the association of signal with cellular morphology. The diagnostic sensitivity of NISH is comparable with that of PCR where productive viral infection occurs, although it appears less sensitive when more minor cytological abnormalities are present. Nevertheless, NISH and PCR analysis provide complementary information and the application of both methods to the investigation of HPV infection is of greater value than either technique alone.

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