

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

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Abstract

Aims: To determine the relative diagnostic performance of non-isotopic in situ hybridisation (NISH) and a dot-blot assay for detecting human papillomavirus (HPV) on exfoliated cervical cells; and to correlate the results with cytopathological assessment.

Methods: Cervical smears and cytological samples were obtained from 122 patients during the same clinical examination and the presence of HPV sequences determined by NISH and dot-blot analysis, respectively.

Results: Dot-blot analysis gave an autoradiographic signal in 15 of 121 (12.4%) cases, while NISH detected viral genomes in 38 of 114 (33.3%) cases. Even in the presence of koilocytosis, where vegetative replication of the virus occurs, NISH was positive in over twice as many cases as dot-blot analysis (NISH 90%, dot-blot 40%), while in smears within normal cytological limits, where the viral copy number is likely to be considerably lower, the differences were more striking (NISH 31%, dot-blot 5%).

Conclusions: These data show that NISH on cytological smears is more sensitive than a standardised dot-blot hybridisation assay for detecting HPV infection in cytological material and is therefore a more appropriate screening tool.

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Infection with certain types of human papillomavirus (HPV 16, 18, 31, 33, 35, 45, 52, 56) has been increasingly implicated as an early event in the natural history of high grade squamous cervical dysplasia (CIN 2-3) and invasive carcinoma.¹ Women with cervical HPV infection may therefore be at greater risk of developing preinvasive and invasive squamous epithelial lesions.¹ Standard histopathological and cytopathological testing does not, however, detect non-productive viral infection, or the presence of integrated virus.²⁻⁴ This can only be demonstrated by nucleic acid hybridisation. Consequently, exfoliated cervico-vaginal cells have been tested extensively for the presence of HPV DNA in clinically normal and abnormal population groups.⁵⁻¹⁷ In these studies, the presence of HPV DNA in cells or DNA obtained by cervical swab or lavage has been assessed by a variety of molecular techniques, including filter hybridisation,⁵⁻⁸ non-

isotopic in situ hybridisation (NISH)⁹⁻¹² and PCR.¹⁴⁻¹⁷ In situ hybridisation has the advantage over other methods in that it permits unequivocal localisation of HPV genomes to epithelial cells and thereby obviates the risk of false positive results due to laboratory or clinical contamination.

To date, only one diagnostic test is commercially available for HPV screening and typing. This is a filter hybridisation assay, based on the use of radiolabelled [³²P] RNA probes. The sensitivity of this test has not previously been compared with NISH. The aims of this study were twofold: first, to assess the relative diagnostic performance of NISH and the dot-blot hybridisation assay and second, to correlate these data with the cytopathological assessment. To address these issues cervical samples were collected from 122 women undergoing routine gynaecological examination. The samples were tested for the presence of HPV by dot-blot and NISH in separate laboratories and the results evaluated double blind for each cytological diagnostic group.

Methods

Women (n = 122) undergoing routine gynaecological examination were recruited from two sites: Greenville, North Carolina and San Diego, California, USA between March and May 1990. A cervical swab was taken and suspended in medium provided in the dot-blot sample collection kit (ViraPap, ViraType, Digene Diagnostics, Inc, Silver Spring, Maryland, USA). A cervical smear was taken at the same time from each patient and fixed immediately in the standard way. NISH was performed on the smear while the suspended cells, from the swab, were assayed for the presence of HPV and HPV type by the dot-blot assay. After NISH was performed, smears were counterstained with haematoxylin and assessed both cytopathologically and for the presence of NISH signal.¹¹ Cytopathology, NISH (Oxford University) and dot-blot hybridisation (Roche Biomedical Laboratories) were performed double blind and the results compared.

DOT-BLOT FILTER HYBRIDISATION

Cell disruption, denaturation, and immobilisation of DNA on to nylon filters, hybridisation, and detection were carried out according to the manufacturer's instructions. Briefly, the procedure was as follows: specimens were incubated with sample preparation reagent at 37°C for one hour to complete cell lysis. DNA denaturation was achieved by incubation in

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alkaline solution. The DNA was bound to a nylon filter under vacuum and the membrane transferred to a reaction tray, covered with pre-hybridisation buffer and incubated at 60°C for 30 minutes. The hybridisation mix contained a cocktail of ³²P-RNA transcripts to HPV types 6, 11, 16, 18, 31, 33 and 35. The membrane was hybridised at 60°C for two hours and non-specifically bound probe was removed by RNase treatment and low and high stringency post-hybridisation washes. Autoradiography was then carried out at -70°C for 48-72 hours and the presence of HPV DNA in the sample was determined by the presence of an autoradiographic signal. Samples positive by dot-blot (ViraPap) were further HPV typed by ViraType assay, which differs only in that three replicate membranes are hybridised with separate cocktails of complementary radiolabelled probes (HPV 6/11; 16/18 and 31/33/35).

NISH ON CERVICAL SMEARS

In situ hybridisation was performed on routine cervical smears, as previously described.¹¹ Briefly, smears were washed in freshly prepared methanol/acetic acid (3:1 = v/v), fixed in 4% paraformaldehyde (w/v) in PBS (PBS = 10 mM phosphate, 150 mM NaCl, pH 7.4), endogenous peroxidase activity blocked in peroxide/azide and nucleic acids unmasked using 1 µg/ml proteinase K. Smears were then postfixed in paraformaldehyde, washed in PBS and air dried. 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 containing 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 400 000 molecular weight, 0.2% (w/v) Ficoll 400 000 molecular weight 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.¹⁸ After hybridisation, smears were washed twice in 4 × SSC and soaked in blocking agent (TBT) comprising 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. Smears were sequentially incubated in monoclonal anti-digoxin (Sigma UK), biotinylated rabbit anti mouse F(ab')₂ fragment (Dako UK), and avidin peroxidase conjugate in TBT containing 5% (w/v) non-fat milk (Cadbury UK).¹⁹ A red NISH signal was developed by incubation in 3-amino-9-ethylcarbazole, H₂O₂ (Zymed USA) for 10 minutes. The reaction was stopped by washing in water smears counterstained progressively with haematoxylin for 10-15 seconds and mounted in glycerol jelly.²⁰

CYTOPATHOLOGICAL ASSESSMENT OF NISH SMEARS

After NISH, the smears were counterstained with haematoxylin, cytologically assessed, and divided into five diagnostic groups. The first group were assessed as being within normal limits. "Minor" wart virus infection was diagnosed on the basis of the following morphological criteria: squamous cells arranged in a concentric manner with cohesive rounded margins and abnormal nuclei. When these changes were accompanied by koilocytosis, a cytological diagnosis of wart virus infection (WVI) was made. The final diagnostic group contained dyskaryotic smears showing nuclear enlargement with a high nucleolar:cytoplasmic ratio.

The two methods were compared using either the χ^2 test for independent samples (where all expected values were greater than 5) or a two-tailed Fisher's exact probability test.²¹

Results

DOT-BLOT ANALYSIS

Dot-blot hybridisation gave an autoradiographic signal (fig 1) in 15 of 121 (12.4%) cases. One case was not analysed due to an excessively high red cell content. HPV 6/11 were found alone in one case, HPV 16/18 alone in three cases, and HPV 31/33/35 alone in nine cases. Two cases produced multiple signals: in one HPV6/11 and HPV31/33/35 were present and in the other HPV16/18 and HPV31/33/35. Correlation of these results with the cytopathological diagnosis (table 1) demonstrates that four of 81 (5%) smears

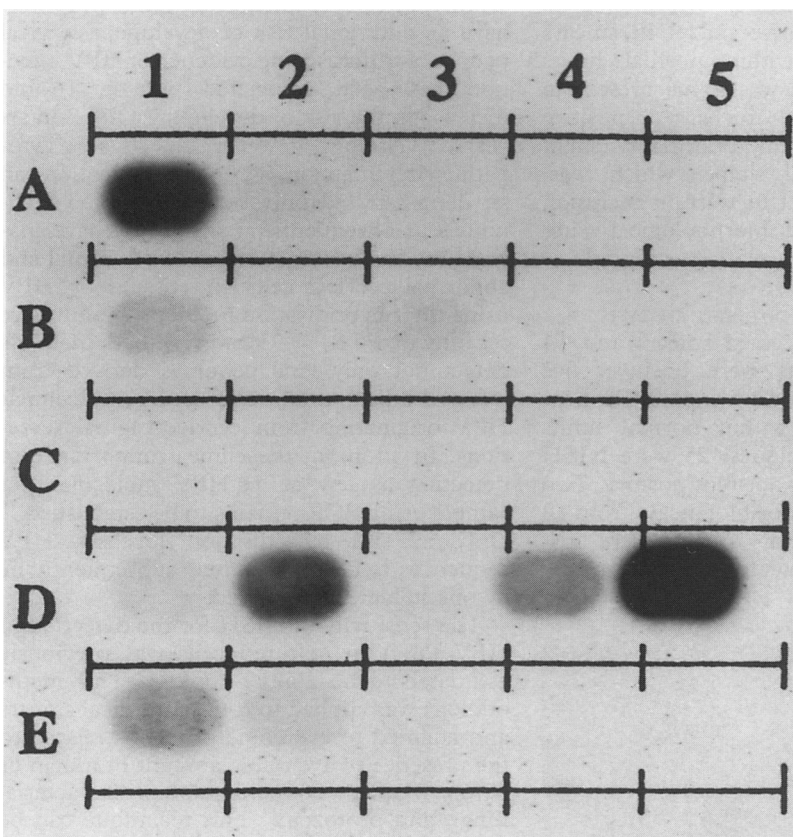


Figure 1 A representative autoradiograph of the dot-blot assay for HPV. The dot-blot membrane includes a high positive (A1), low positive (B1), and negative control (C1). Samples that produce an image equal to or greater than the low positive control are considered positive for one or more viral types from the group HPV 6, 11, 16, 18, 31, 33, and/or 35. Examples of positive specimens are shown in rows D and E. Low positive control = 2×10^4 disrupted HeLa cells; high positive control = 1×10^5 disrupted HeLa cells; negative control = 5×10^5 disrupted HTB-31 cells.

Table 1 Cervical cytopathology and HPV detection by dot-blot and NISH

Cytopathology	n =	Dot-blot	NISH	p Value
Normal	82*	4 (5%)	25 (31%)	p < 0.001†
Inflammatory	12	4 (33%)	2 (17%)	p > 0.05‡
Minor wart virus changes	6	1 (17%)	1 (17%)	
Wart virus infection	10	4 (40%)	9 (90%)	p > 0.05‡
Mild/moderate dyskaryosis	4	1 (25%)	1 (25%)	
Not determined	8	1 (12.5%)	ND	

*In one case with normal cytopathology, which was negative by NISH, dot-blot analysis was not possible due to an excessively high red cell concentration in the sample.

ND Not determined due to excessive non-specific substrate deposition.

† χ^2

‡Fisher's exact probability test (two-tailed).

within normal limits, four of 12 (33%) inflammatory smears, one of six (17%) minor wart virus infection, four of 10 (40%) WVI smears and one of four (25%) dyskaryotic smears were dot-blot positive. In eight cases cytopathological assessment was not possible due to non-specific substrate deposition: one of these cases was dot-blot positive for HPV 31/33/35.

NISH ANALYSIS

In eight cases the NISH result was uninterpretable due to non-specific substrate deposition. Using a cocktail of HPV probes (6, 11, 16, 18, 31, 33) on the remaining 114 cases, NISH detected viral genomes in 38 cases (33.3% (fig 2)). HPV DNA was found in 25 of 82 (31%) smears within normal cytopathological limits, two of 12 (17%) inflammatory smears, one of six (17%) minor wart virus infection, nine of 10 (90%) smears showing diagnostic features of WVI and in one of four (25%) dyskaryotic smears. Morphological analysis of NISH positive nuclei in smears diagnosed as being within normal limits showed that in no case was signal present in morphologically normal nuclei. All positive cells showed minor nuclear abnormality (slight enlargement, abnormal shape) which was, nevertheless, insufficient to warrant inclusion in a defined abnormal morphological category.

COMPARISON OF NISH AND DOT-BLOT ANALYSIS

Overall, 38 NISH processed smears and 14 corresponding dot-blot assays were positive; five cases were dot-blot positive and NISH negative. Of 81 smears within normal limits analysed by both techniques, 25 were NISH (29.6%) and four (5%) dot-blot positive. Two NISH smears and four dot-blot assays from 12 patients with inflammatory smears were positive. Of six smears showing minor diagnostic

Table 2 Correlation of NISH and dot-blot hybridisation

NISH	Dot-blot hybridisation	
+	+	-
	9	29
-	5	70

cytological criteria of HPV infection, two were positive by NISH and one by dot-blot. Of 10 smears containing koilocytes, nine (90%) were positive by NISH and four (40%) by dot-blot. Of four cases with evidence of dyskaryosis, one (25%) was both NISH and dot-blot positive.

The correlation of NISH and dot-blot hybridisation, regardless of cytopathological diagnosis, is shown in table 2. Five cases were dot-blot positive and NISH negative; and 29 cases were NISH positive, dot-blot negative.

In each dot-blot assay the controls included by the manufacturers gave the expected autoradiographic signal. Controls in all NISH experiments included CaSki cell smears (which contain integrated HPV 16²²); and cytologically normal cervical smears (from a separate cohort). These were hybridised with HPV 16 and total human DNA, respectively.²³ Multiple nuclear signals were obtained in CaSki cells²⁰ and total human DNA labelled every nucleus in control smears.

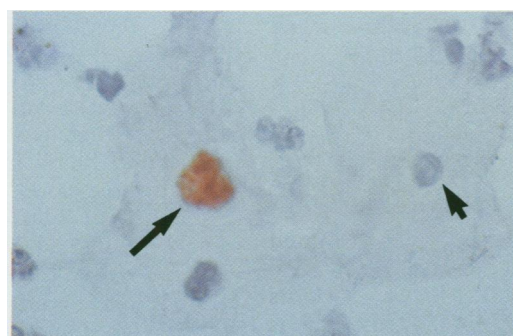
Discussion

Women infected with specific HPV types may have an additional risk of developing cervical neoplasia. Microscopic evidence of HPV infection has been associated with a 16-fold increase in the risk of developing CIN 3 in six years.²⁴ Cytology, however, detects only cytopathic viral infection, while latent infection can be demonstrated only by hybridisation techniques.² Consequently, many groups have analysed the frequency of infection of normal and abnormal cervical cells and tissues by HPV using filter hybridisation methods⁵⁻⁸ and more recently by PCR.¹⁴⁻¹⁷ However, these methods detect not only viral genomes derived from infected cells, but may also detect extracellular HPV originating from cervico-vaginal secretions. In addition, the clinical importance of detecting as few as 10 HPV molecules per sample using PCR remains to be established.¹⁶ Only in situ hybridisation localises HPV sequences to cells which may subsequently be cytopathologically assessed.

The sensitivity of NISH for the detection of HPV DNA in cultured cells was previously estimated to be 2.5-12 copies.¹⁹ This methodology was applied to routine cervical smears and adapted to overcome problems related to the presence of a variable amount of mucin or to the frequent contamination with bacteria, fungi, and protozoa.¹¹ This technique can be performed using basic laboratory equipment within one working day and therefore has a potential role in routine cervical screening.

At present, only one dot-blot diagnostic test is commercially available for HPV detection in exfoliated cervical cells. This is a filter hybrid-

Figure 2 A routine cervical smear was hybridised with a cocktail of probes for HPV 6, 11, 16, 18, 31 and 33 and detected as described. HPV signal is present within an epithelial cell nucleus (arrows). Note the absence of signal in neutrophil polymorphs and another epithelial cell nucleus (arrowhead).



isation assay based on the use of radiolabelled [^{32}P] HPV probes. These hybridise with the target HPV DNA which is derived from the lysis of the infected cell and linked to a supporting filter matrix. Following hybridisation the presence of bound probe is determined by autoradiography. The sensitivity of this assay has been estimated at 50 000 copies of HPV (DiGene Diagnostics Inc; Package insert, 1991). In the present study dot-blot assay and NISH were performed on cervical cell specimens obtained from the same patients at the same time and the results correlated with each other and with cytopathological assessment. The results show that dot-blot hybridisation is less sensitive than non-isotopic in situ hybridisation. Even in the presence of koilocytosis, where vegetative replication of the virus occurs, NISH was positive in over twice as many cases as the dot-blot hybridisation assay. In smears within normal cytological limits, where the viral copy number is likely to be considerably lower, the differences were more striking, with NISH detecting the viral genome in 31% of cases compared with only 5% by dot-blot analysis ($p < 0.001$). This discrepancy between the two techniques may reflect differences in absolute sensitivity (2.5–12 copies per cell for NISH; 50 000 copies for dot blot hybridisation), but may be attributable in part to the detection of additional types by the whole genomic HPV probes used in the NISH assay (Herrington *et al*, Anderson *et al*, unpublished observations).

The correlation of NISH and dot-blot results from individual cases shows that NISH positive, dot-blot negative discordant cases are more common ($n = 29$) than dot-blot positive NISH negative cases ($n = 5$). This finding excludes the possibility that sampling error is the sole explanation for the overall differences between NISH and dot-blot analysis. Of the five dot-blot positive, NISH negative cases, three were typed as HPV31/33/35 by dot analysis, and two as HPV16/18. Although it is possible that the three HPV31/33/35 positive cases may be infected with HPV35 (which was not present in the cocktail of NISH probes), this discordance may be due to either sampling error or technical failure affecting both systems.

The data obtained with smears within normal cytological limits indicate that HPV infection is underestimated by regular cytopathological criteria. When the positive cells in these smears were analysed critically, minor nuclear abnormalities were always present. These varied from slight nuclear enlargement to abnormalities of nuclear shape, but, in no case was HPV signal present in a completely normal nucleus. This concurs with both a recent study from this laboratory¹¹ and the fact that HPV sequences have never been shown in normal epithelial cell nuclei by in situ hybridisation.²⁵ In a recent study from a separate cohort of patients from a sexually transmitted disease (STD) clinic, the prevalence of HPV sequences as determined by NISH in patients with cytologically normal parallel smears was 41%.¹⁰ This concurs with data derived by PCR

analysis.^{16 17} Thus the apparently high prevalence of HPV sequences in patients with apparently normal cervical smears is probably due in part to sampling error between the Papinacolaou stained smear and the sample used for molecular analysis, and in part to the failure of screening cytopathology to detect minor nuclear changes associated with HPV infection. The management of women with human papillomavirus DNA without cytological evidence of a lesion therefore presents clinical difficulties. Such patients are at increased risk of developing preneoplastic and invasive lesions and close follow up screening is required.²⁶

In conclusion, we have shown that NISH on cytological smears has potentially greater diagnostic sensitivity than a standardised dot-blot assay for detecting HPV infection. This NISH method is also suitable for a large scale screening programme. It does not require any change in the Papinacolaou smear sampling procedure, being performed on a second specimen collected in the same way. Modification of the procedure has allowed low and high risk types to be differentiated on individual routine cervical smears.¹⁰ Another advantage is that the same cytotechnician can interpret both the molecular and cytological result. However, to assess the relation between HPV and cervical cancer, data are required on the prevalence of the virus, together with the natural history of the associated lesions on follow up. As a tool to answer these questions, NISH may be used as a sensitive, specific and routinely applicable screening technique.

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