Detection of low copy human papilloma virus DNA and mRNA in routine paraffin sections of cervix by non-isotopic in situ hybridisation

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SUMMARY In analysing human papilloma virus (HPV) infection of the cervix in formalin fixed paraffin sections by non-isotopic in situ hybridisation two main problems were found: detachment of sections from the glass during hybridisation and probe detection; inadequate sensitivity and inability to assess sensitivity of the in situ procedure. The first problem was investigated by assessing the efficiency of various tissue adhesives individually and in combination. The second problem was addressed by optimising conditions for DNA unmasking, hybridisation, and biotinylated probe detection. Sensitivity of the final in situ procedure developed was assessed by using the detection of pHY2.1 repeats as a built-in control. Extrapolation of data showed that less than 10 copies of HPV DNA can be visualised by these procedures. HPV nucleic acid, mainly in the form of DNA, was detected not only in koilocytic nuclei but also in suprabasal cells in condylomas and CIN lesions. HPV mRNA was also visualised in the cytoplasm (and probably also nuclei) of the same cell types. These non-isotopic in situ procedures give results comparable to those obtained with radiolabelled probes, but they are less time consuming and provide better morphological resolution.

Invasive squamous cell cancer of the cervix is associated with human papilloma virus (HPV) types 16, 18, and less commonly 31, 33, 39 and others, while benign warts of cervix are typically associated with HPV 6 and 11.¹ Cervical dysplasia (CIN) also contains HPV DNA sequences of various types. Filter hybridisation studies in general indicate that HPV 6 and 11 are present in the milder forms of CIN (I and II), while HPV 16 and 18 occur in more severe grades (CIN III)²⁻⁴ as well as in invasive cancers. This generalisation, however, is not absolute. HPV 16^{5,6} has been detected in mild dysplasia and in clinically normal women¹ while HPV 6-like sequences have been reported in CIN III and in invasive carcinoma.⁷

CIN, as defined by morphological criteria, is a heterogeneous group of conditions evidenced by their clinical behaviour. Severe dysplasias seem to persist or progress whereas milder dysplasia can regress. Like all biological generalisations, however, there are common exceptions; severe dysplasia may regress without treatment,⁸ while some mild dysplasia may progress.⁵ Because of the general association of HPV 16, 18, 31 and 39 with invasive cancer, and HPV 6 and 11 with benign lesions, it is conceivable that the likely clinical

outcome of CIN grades may be more predictable when morphology is combined with specific HPV typing.

Complete HPV particles can be identified by electron microscopy (EM), and HPV genus specific immunohistochemistry recognises "late" HPV proteins.⁹ Neither technique identifies non-productive HPV DNA. Capsid antigens are usually only expressed in superficial cells such as koilocytes of non-invasive lesions and not in basal cells. A few cells in invasive cancer may be antigen positive.⁷ Filter and in situ DNA hybridisation do not have these advantages.¹⁰ Of these, filter hybridisation is reckoned to be more sensitive for HPV DNA detection. It is estimated that in situ hybridisation with non-isotopic probes detects only about 800 copies of virus per cell.¹⁰ There are also several methodological problems in combining in situ hybridisation analysis with histopathological interpretation of biopsy specimens. Among these are the tendency for sections to detach from slides during in situ hybridisation, the relative insensitivity of non-isotopic methods, and the time required for in situ analysis (particularly with radioisotopic probes).¹¹

Previous work on non-isotopic methods for in situ nucleic acid detection has shown that multiple copy

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DNA sequences can be visualised in frozen¹² and paraffin sections¹³ and on chromosome spreads.¹⁴ More recently, single copy genes have also been visualised on chromosomes.¹⁵ This paper investigates the optimal conditions for tissue adhesion, DNA unmasking, denaturation and hybridisation, and the sensitivity of biotinylated probes in detecting HPV nucleic acids in routine formalin fixed paraffin embedded cervical biopsy specimens. We estimate that the method reported detects fewer than 10 copies of HPV DNA per cell and also shows HPV mRNA not only in koilocytes but also in suprabasal cells in condylomas and CIN lesions.

Material and methods

Formalin fixed, paraffin embedded cervical biopsy specimens (table) from our routine surgical file were used in this study. A skin wart biopsy specimen from a man was used as a control. The only criteria in selection of cervical biopsy specimens for the study was morphological evidence of wart virus infection (koilocytes etc). Blocks were 1–4 years old. All sections were mounted on multiwell slides (Henley, Essex).

SECTION ADHESION, PROTEOLYSIS ETC

Sections (5 µm thick) were floated on to 0.1% aqueous Elmers' "Glue-All" (Borden Inc, USA) at 45°C in a water bath and mounted on multiwell slides coated with 0.1% (w/v) poly-d-lysine hydrobromide, molecular weight 300–700 kilodaltons, (Sigma, UK) in 0.1% (v/v) Tween 20 (BDH, UK). Slides coated in poly-l-lysine hydrobromide,¹³ poly-d-lysine hydrobromide, and Elmers' "Glue All" were tested individually for section adhesion efficiency. Multiwell slides were also coated with aminopropyltriethoxysilane, which was originally used for chromosome spreading.¹⁶ These slides were first washed in 2% Decon, rinsed in H₂O, then acetone, and immersed in 2% aminopropyltriethoxysilane in acetone for five minutes, washed in H₂O and dried overnight at 22°C. Batches of 300 slides were conveniently prepared at

one time and stored on the bench at 22°C for weeks before use. Sections mounted on slides coated with these different adhesives were dried at 37°C for 30 minutes, baked sequentially at 75°C for 60 minutes, and overnight at 60°C. Slides required urgently can be used after the 75°C baking step. Sections were stored at 22°C for up to 36 weeks.

Unmasking of target DNA was performed with pepsin hydrochloric acid¹³ or pronase.¹⁷ Unmasking with 0.4% pepsin, 3200 units/mg protein (Sigma UK, No P6887), in 0.2 M hydrochloric acid at 22°C or 37°C for 15, 30, and 60 minutes, respectively, was explored. In practice it is important to dissolve 400 mg of pepsin with gentle agitation in 96 ml of distilled H₂O at 22°C and then slowly add 4 ml 5 M hydrochloric acid to a final concentration of 0.2 M hydrochloric acid.

DNA probes pBR322, pHY2.1, HPV 2, 6K2.1, 6amp2, 11, 16 and 18 were nick translated with biotin-dUTP with an 11 carbon linker arm by standard procedures, described in detail elsewhere.¹⁴ Nick translation reagents were obtained from Amersham (UK). Biotin incorporation into each probe varied from 15–30% and nick translated probe ranged in size from <100–1500 bases. The predominant species in each was 200–400 bases.

Denaturation of both target and biotinylated probe was performed simultaneously. Denaturation temperatures and times ranging from 70–100°C and 10–30 minutes, respectively, were tested in a hot air convection oven. The slides in humidified Terasaki plates were placed on a solid stainless steel plate within the oven to obviate the inevitable temperature drop which occurs when the oven is opened.

Hybridisation of probe to target was conducted at a constant temperature of 42°C in a conduction hot air oven. The periods of hybridisation tested varied from one to sixteen hours.

Both low and high stringency washing schedules (two to three changes, 10 minutes each change) for post-hybridisation washes were used. Low stringency washing was conducted at 22°C, in 4 × SSC. High

Table Tissue diagnosis, HPV type, and intensity of pHY2.1 signal v fixation time

Case No	Diagnosis	Fixation time (days)*	HPV type†	pHY2.1 intensity
1	Skin wart	4	2	++
2	CIN II with wart virus change	1	18	++
3	CIN II with wart virus change	1.5	11	ND
4	CIN I with wart virus change	1.5	18	++
5	Condyloma	1.5	11	++
6	Condyloma	1.5	11	+++
7	Wart virus infection	4	6	+

*All tissues were fixed in 10% neutralised formal saline.

†All biopsy specimens were probed with HPV 2, 6, 11, 16, 18, pBR322 and pHY2.1. pBR322 did not bind to any biopsy specimen. In addition, however, biopsy No 6 gave signals with HPV 6 and 16 and biopsy No 7 signals with 11 and 16; these latter reactions were diminished, but remained evident after washing at 65°C in 0.1 × SSC.

stringency washing followed the low stringency washing schedule and was done in $0.1 \times \text{SSC}$ at temperatures ranging from 42–65°C; $1 \times \text{SSC} = 0.15 \text{ M}$ sodium chloride containing 0.015 M sodium citrate, pH 7.4.

Sensitivity of detection of bound probe was assessed with several reagents: streptavidin-biotinylated horseradish peroxidase (HRP) conjugate (Enzo Biochem Inc, USA); avidin (modified)—HRP conjugate (Dako, UK); streptavidin-biotinylated alkaline phosphatase (AP) conjugate (Enzo, USA and BRL, UK); avidin (modified)-AP conjugate (Dako, UK); silver amplified indirect immunoperoxidase procedure for biotin.¹² All reagents including silver reagents were stored at 4°C for up to five months.

HRP conjugates diluted 1/200 in PBT (5% bovine serum albumin, w/v, (Sigma, UK No A7906), 0.1% triton X 100 (v/v), 0.1 M sodium chloride in 0.1 M phosphate buffer pH 7.4), were reacted with sections for 30 minutes at 37°C, in a moist chamber. HRP in bound conjugates and immunoperoxidase complexes was developed with diaminobenzidine-hydrochloric acid (Polysciences, UK) containing 0.01% H_2O_2 (BDH, UK) and amplified with gold and silver.¹²

AP conjugates were diluted 1/50, 1/200, and 1/400 in PBT or Tris¹⁷; dilution in PBT was more sensitive. Sections were incubated with AP conjugates at 22°C or 37°C for five to 60 minutes. AP was shown by incubation with either AP substrate Kit-1, SK-500, (Vector Laboratories Inc, UK), or nitroblue tetrazolium (NBT) containing 5-bromo-4-chloro-3-indolylphosphate (BCIP)¹⁸ at 22°C for five to 30 minutes. Sensitivity of each detection system was assessed subjectively by observing intensity and number of cells stained.

IN SITU HYBRIDISATION

Having tested the variables above, the best conditions for in situ hybridisation and probe detection are given below. Sections were dewaxed in two changes of xylol, (10 minutes each change), washed in 99% ethanol (10 minutes each change), and washed in running tap water for five minutes. Human and HPV nucleic acids were unmasked by exposing sections to 0.4% pepsin in 0.2 M hydrochloric acid for 15 minutes at 22°C. Sections were washed in tap water (five minutes), rinsed in distilled water and dehydrated through 99% ethanol (two changes of five minutes each), and air dried. Aliquots (9 μl) of hybridisation mix,¹⁴ containing 10 ng of biotinylated probe, were added to wells on multislot slides, covered with 14 mm in diameter glass coverslips and placed in sealed damp Terasaki plates. Sections from each case (table) were hybridised with biotinylated HPV 2, 6K2-1, 6amp2, 11, 16 and 18. pBR322 and hybridisation mix only—minus probe—were used as

negative controls on all slides. The sections and probe were simultaneously denatured for 15 minutes at 95°C and hybridised for two hours at 42°C. Coverslips were removed by soaking the slides in $4 \times \text{SSC}$ at 22°C for five minutes. Thereafter, the slides were washed at low stringency in three changes of $4 \times \text{SSC}$ at 22°C for 10 minutes. If high stringency washing was required the latter protocol was followed by two to three washes in $0.1 \times \text{SSC}$ at 65°C, 10 minutes each wash, followed by a five minute wash at 22°C in $4 \times \text{SSC}$. After these washes the sections were soaked in PBT at 22°C for 10 minutes.

The methods of choice for visualising hybridised biotinylated probes are avidin (modified)—AP conjugates (Dako, UK) and silver amplification of immunoperoxidase reaction products. Avidin-AP was diluted 1/200 (protein concentration = 3.5 mg/ml) in PBT and incubated with sections at 22°C for 10–30 minutes. Unbound conjugate was removed by washing slides for five minutes in 0.1% Tween 20 in PBS, rinsing sequentially in PBS and substrate buffer¹⁸ (pH 9.6), and incubating in NBT-BCIP¹⁸ for five to 30 minutes at 22°C in the dark. The reactions were terminated by rinsing thoroughly in tap water and immersing in PBS for five minutes. The final preparations were rinsed in distilled water, air dried, and mounted in glycerine jelly; some slides were counterstained with a weak Meyer's haematoxylin and eosin stain before mounting. Silver amplification reaction of immunoperoxidase reactions was as described¹²; these slides were counterstained with haematoxylin and eosin and dehydrated in alcohol, cleared in xylene, and mounted in DPX (Raymond Lamb Co, UK).

To determine whether the signals, observed after in situ hybridisation, were due to DNA or RNA, sections were digested with RNase or DNase before hybridisation. RNase A (Sigma, UK) was dissolved in $2 \times \text{SSC}$ (10 mg/ml), boiled for 10 minutes, and diluted to 100 $\mu\text{g}/\text{ml}$ in $2 \times \text{SSC}$; RNase T1 (Boehringer, West Germany) was then added to a final concentration of 100 units/ml. DNase 1 (Boehringer, West Germany) was dissolved at 200 $\mu\text{g}/\text{ml}$ in 0.01 M Tris-hydrochloric acid (pH 7.5), containing 0.01 M magnesium chloride₂. One hundred and thirty μl of the RNase or DNase solutions were placed over each multislot and the sections incubated for 30 minutes at 37°C. Sections were washed in $2 \times \text{SSC}$, rinsed in distilled water, 100% ethanol, and dried in air at 22°C before hybridisation.

Results

Although an HPV signal was seen in all six cervical biopsy specimens and one skin wart, the intensity of the reaction with pHY2.1 diminished with prolonged

Fig 1 Condyloma probed with HPV 11. All koilocytes are labelled as are many suprabasal cells. Cytoplasmic hybridisation is indicated by arrows. Avidin (modified) alkaline phosphatase detection. Same detection system was used in figs 2-6.

Fig 3 CIN II lesion probed with HPV 18. Suprabasal cell nuclei and cytoplasm show hybridisation signal.

Fig 2 Same case in fig 1 except that the section was counterstained with haematoxylin and eosin after hybridisation. Cytoplasmic hybridisation with HPV 11 is clearly evident (arrows).

Fig 4 CIN II lesion probed with HPV 11. Nuclear and cytoplasmic signals are present in cells close to surface of epithelium.

fixation time. Two condylomas were HPV 11 positive. One of these, however, (case 6) also gave reactions with HPV 6 and 16. A triple signal was also seen in case 7 with HPV 6, 11, and 16. In cases 6 and 7 the strongest signal was obtained with HPV 11 and 6, respectively. The signals with the other probes were diminished, but not abolished, by post-hybridisation washes at 65°C in 0.1 × SSC. It is likely, therefore, that there were triple infections in these two cases. Those reactions which were decreased after high stringency washings, however, may indicate lack of complete homology between probe and tissue HPV. The three cases of CIN with associated wart virus morphology gave a signal only with HPV 18 or 11. There were no morphological features that were specifically associated with any single HPV type.

The nuclei of most koilocytes (when the nuclei were present in the plane of section examined) gave a signal with at least one HPV probe (figs 1 and 2). Nuclear HPV signals were also seen in suprabasal cells (figs 1

and 2). The in situ detection procedures are compatible with subsequent haematoxylin and eosin staining (fig 2). Figs 1 and 2 also show that 3-aminopropyltriethoxysilane is an excellent adhesive.

Cytoplasmic hybridisation with HPV probes was observed in four cases (cases 2, 3, 6, 7). Foci of cytoplasmic hybridisation occurred in the middle or upper third of the epithelium (fig 1) and in some suprabasal cells (fig 3). As shown in figs 4 and 5 the cytoplasmic signal is abolished by RNase digestion, indicating that this signal represents HPV transcripts. Most cytoplasmic sites of hybridisation resisted digestion with DNase, further substantiating this conclusion (fig 6). The nuclear HPV signals in most cells were entirely abolished by DNase digestion (fig 6), indicating that these were largely due to HPV genomes. In some cells a nuclear signal was still present after DNase digestion indicating that small amounts of HPV RNA was present in some nuclei (fig 6). Addi-

Fig 5 Parallel section to that shown in fig 4. This section was digested with RNase A and RNase T1 before hybridisation with HPV 11. Cytoplasmic signal has been abolished in most cells. There is residual cytoplasmic signal, however, in two cells (arrows).

Fig 6 Parallel section to that shown in fig 4 and 5. This section was digested with DNase before HPV 11 probing. Nuclear signal is abolished or grossly diminished in all cells. Cytoplasmic signal is present in some cells, indicating presence of RNA (arrows).

tionally, the cytoplasmic signal in some cells resisted RNase digestion (fig 5) indicative of HPV genomes in the cytoplasm.

pHY2·1 hybridised to nuclei in all cases examined, but was weak in case 7 where the biopsy specimen had been fixed for four days; this was due to prolonged fixation of the specimens and can be overcome by increased proteolysis (unpublished observations). In case 1 (male skin biopsy specimens) the hybridisation signal was typical of male cells.¹³ In the female tissues the nuclear reaction with pHY2·1 consisted of multiple dots. Using avidin-AP the dots coalesced (fig 7), making it difficult to count individual dots; in some cells about 10 dots could be visualised. With silver amplification 20–30 dots could be easily counted in most nuclei (fig 8).

pBR322 did not react with any of our cases, and a colorimetric reaction was not generated after hybridising in hybridisation mix alone.

Fig 7 Case 6 probed with pHY2·1. All nuclei are stained. Signal is present as discrete/confluent dots in most cells. Biotinylated DNA was visualised with alkaline phosphatase procedure.

Fig 8 Parallel section to that shown in fig 7 probed with pHY2·1. There are up to 30 dots of positive signal in these nuclei as visualised by silver amplified immunohistochemistry.

Section adhesion was maintained throughout these experiments when 3-aminopropyltriethoxysilane was used as the adhesive. The combined Elmers' "Glue-All"—poly-l-lysine hydrobromide adhesive, although less efficient in section adhesion, is adequate for most purposes. Poly-l- or poly-d-lysine hydrobromide or Elmers' "Glue-All", when used individually, were less efficient than the other adhesives, and sections were often lost or fragmented.

Unmasking of target DNA with 0·4% pepsin in 0·2 M hydrochloric acid at 22°C for 15 minutes was optimal for HPV and detection of autosomal Y repeat sequence. Unmasking with 0·4% pepsin in 0·01 M hydrochloric acid for one hour at 22°C gave a diminished signal with HPV probes and pHY2·1 when compared with the signal intensity obtained by unmasking with 0·4% pepsin in 0·2 M hydrochloric acid. Pronase digestion¹⁷ was less effective than pepsin/0·2 M hydrochloric acid in two respects; the

signal was less intense and the background was higher.

Optimal denaturation of both target DNA and probe was reproducibly obtained when performed for 15 minutes at 95°C. This is the most crucial stage during these in situ hybridisation schedules for the generation of intense signals and must be strictly adhered to. At 95°C sections do not disintegrate or tear as happens with temperatures of 100°C or above.¹⁷

Hybridisation of probe to target DNA for two hours at 42°C was optimal when denaturing at 95°C. The signal was diminished after hybridising for one or 16 hours; the loss of signal was less apparent when hybridisation followed denaturation for 10 minutes at 73°C,¹³ but the signal was much less intense than that obtained after denaturation at 95°C.

Washing at low stringency was adequate for most purposes. When more than one HPV type was shown in a biopsy specimen, the high stringency washing schedule readily differentiated complete from partial homologous binding of HPV probes to target.

Efficiency of detection of bound probe varied with each colorimetric detection system. The avidin (modified)—AP conjugate and silver amplification procedures, however, gave consistent results, with high sensitivity and low background. Optimal results were obtained when the avidin (modified)—AP conjugate was used diluted 1/200 in PBT, for 30 minutes at 22°C, and developed in the NBT-BCIP substrate for 30 minutes at 22°C. High copy numbers of HPV were readily detected after one to two minutes in NBT-BCIP. Silver amplification immunohistochemistry for biotinylated probe detection was more time consuming but more sensitive than avidin-AP.

Discussion

The main technical problem we had at the start of this investigation was loss of sections during hybridisation/probe visualisation. The best tissue adhesive investigated was 3-aminopropyltriethoxysilane. No sections were lost in more than 1000 experiments.

Using the optimal conditions for DNA unmasking, denaturation, hybridisation, and biotinylated probe detection, pHY2-1 repeats could be visualised in cervical cells. There are 100–200 copies of this repeat in female cells and each repeat is 2.1 Kb.¹⁹ As 10–30 dots were seen in each nucleus, using alkaline phosphatase and silver amplification, respectively, each dot would correspond to between seven and 20 repeats if it is assumed that these are evenly and equally dispersed throughout an interphase nucleus. Because the HPV genome is four times larger than pHY2-1 repeats, it would follow that the level of sensitivity of the procedure for HPV detection is of the

order of two to five copies/cell, but in practice we assume it is less than 10. Previous assays of sensitivity of in situ non-isotopic procedures have been based on extrapolation from cultured cells infected with known numbers of copies of SV40 (a virus similar to HPV).¹⁰ These authors concluded that their sensitivity of in situ HPV detection was 800 copies of HPV/cell. This experiment is open to the criticism that cultured cells do not fix in the same way as solid biopsy specimens because of permeability and diffusion factors. Additional methods for determining sensitivity is the correlation of results of filter hybridisation with those of in situ hybridisation on 2 aliquots of the same biopsy specimen.¹⁰ In this type of calculation factors such as variability of distribution of HPV within the tissue, which can be large, cannot be compensated for; nor can the fact that some cells obviously contain very large amounts of HPV nucleic acid and many others none (figs 1 and 2). Although it has been stated that dot hybridisation is more sensitive than in situ hybridisation,¹⁰ this statement ignores the relative weights of a single section v a whole punch biopsy specimen. A 5 µm section from a typical punch biopsy specimen (6 × 2 × 2 mm) used here weighed 25 µg, and the whole biopsy specimen processed to xylol (for dot hybridisation) 10.1 mg. It is therefore logical to use as the yardstick of sensitivity, an in-built tissue control such as pHY2-1, or preferably a single copy gene. As single copy genes can be visualised on chromosomes by non-isotopic methods¹⁵ it should be possible to achieve this sensitivity in paraffin sections. In this laboratory each cervical biopsy specimen probed with HPV is only considered to be of acceptable standard if the reaction with biotinylated pBR322 is negative and pHY2-1 is readily seen. These criteria eliminate non-specificity and establish sensitivity.

These morphological results show, in practical terms, the sensitivity of non-isotopic in situ hybridisation by virtue of the labelling of suprabasal cells and this has also been recorded in isotopic methods. It has been shown here, however, that HPV mRNA is detectable by non-isotopic methods. This would be difficult to visualise on autoradiographs because of the track length of tritium (1 µm in water) and the interference of microscopic resolution by the autoradiographic film. There are two further points of biological interest—namely, HPV DNA was present in the cytoplasm of some superficial cervical cells and this (fig 5) could be interpreted as virus in transit out of or into the nucleus/cell. In some cells the HPV signal in nuclei is a composite of DNA and RNA (fig 6) and not only DNA.

From the purely diagnostic viewpoint it is clear from this and other studies that HPV is present in many cells other than koilocytes. It follows, therefore, that the absence of koilocytes does not necessarily

rule out HPV infection. The triple HPV signal in three cases probably indicates five triple HPV infection. As the signal with two of the three HPV probes diminished with high stringency washing, however, this may well indicate that the signals which diminished represent viruses similar to, but not entirely homologous with, HPV 11 and 16. This conclusion is further substantiated by the fact that there was only a single HPV signal in all of the other cases where only single HPV types were observed.

The sensitivity of the methodology described here should now enable several biological and clinical questions to be answered on archival and prospective formalin fixed paraffin embedded biopsy specimens. The procedures described can be easily completed in one working day, making them amenable for use in routine laboratories.

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