Detection of human papilloma viruses in paraffin wax sections with biotinylated synthetic oligonucleotide probes and immunogold staining

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SUMMARY Human papilloma virus was detected by in situ hybridisation in routinely processed paraffin wax sections using a synthetically produced oligonucleotide probe, end-labelled with biotin, and amplified with anti-biotin-immunogold silver staining (anti-biotin-IGSS). This system proved more sensitive than amplification with streptavidin-biotinylated alkaline phosphatase for detecting human papilloma virus type 16 in cervical tissues. The method was successfully combined with antigen staining for papilloma virus common antigens in skin and genital warts.

This simple and quick method, using non-radioactively labelled synthetic probes, may be useful for the detection of other viruses in stored material and may be suitable for other double staining procedures.

Current methods of in situ hybridisation (ISH) for the detection of viruses rely on probes prepared from cloned nucleic acid, and often make use of expensive radiolabelled probes with limited half-lives and lengthy assay systems which are not readily adaptable to a diagnostic situation. While biotinylated probes are becoming more widely used,^{1,4} alternatives to cloned probes are less well documented. Recently, a method of ISH was described in which human papilloma viruses (HPV) of different types could be detected using specific synthetic oligonucleotides, produced from the known sequence data, as probes.⁵ We report here a modification of that technique, which was more sensitive in the detection of low copy numbers of HPV, as may be the case in cervical lesions. It was also found to be suitable for detection of viral antigen as well as viral DNA using double staining reactions.

Material and methods

Standard sections of formalin fixed, paraffin wax-embedded tissues were mounted on chemically silanated slides⁶ and prepared for hybridisation by pre-treatment with 0.2M HCl and 0.2 mg/ml proteinase K (Sigma) as previously described.⁵ Oligonucleotide probes of 30 nucleotides in length from within the E6

gene of HPV-1a, HPV-6b, and HPV-16 were synthesised on an Oswel Gene Synthesiser and biotinylated using 3'-terminal transferase.⁵

Hybridisation was carried out for two hours at 42°C in high stringency buffer⁴ and the sections washed thereafter in 2 × SSC (300 mM sodium chloride, 30 mM sodium citrate, pH 7.0) three times for five minutes each. Hybridised DNA was initially detected using streptavidin-biotinylated alkaline phosphatase with nitro-blue tetrazolium and bromochloroindolyl phosphate as substrate (BRL DNA detection system, Gibco Ltd; Method 1) according to the manufacturer's instructions, except that streptavidin and conjugate incubations were increased to 20 minutes. Sections were examined without counterstaining after mounting in glycerol-gelatin. An alternative detection system was also tried (Method 2). Mouse monoclonal anti-biotin (Dako Ltd; M743) (1/20 in 5 mM Tris-HCl, pH 7.4, 150 mM sodium chloride (TBS) + 1% bovine serum albumin (BSA) (TBSB)) was applied for 45 minutes at room temperature. After two five minute washes in TBS, non-specific reactions were blocked with 20% normal rabbit serum in TBS for 10 minutes. Twenty five microlitres of rabbit anti-mouse immunoglobulin coated with colloidal gold (Dako Ltd, G385) were added at a dilution of 1/50 in TBSB for one and a half hours at room temperature. The slides were washed in distilled water for 10 minutes and the deposited gold particles were enlarged using the intense II silver enhancement kit (Janssen, KN

Biomedicals Ltd, High Wycombe), based on the immunogold silver staining method (IGSS) originally described by Holgate *et al.*⁷ Silver precipitation was observed under the microscope within 10 minutes and the reaction stopped by immersion in distilled water before "self nucleation" took over to cause high background staining.

In situ hybridisation using cloned HPV-1a, 11, and 16 DNA was carried out using detection Method 1.⁵

For double staining, papilloma virus common antigens were detected before hybridisation. Sections were dewaxed and rehydrated, and endogenous peroxidase was blocked with 3% H₂O₂ in methanol for 15 minutes. After treatment with 0.1% trypsin containing 0.1% CaCl₂ for 15 minutes at 37°C, non-specific-binding of secondary antibody was blocked with TBS + 20% normal pig serum for 10 minutes. Rabbit antiserum to bovine papilloma virus type 1 (BPV-1) disrupted with sodium dodecyl sulphate (Dako Ltd; B580) was applied as the primary antibody (1/20 dilution in TBS + 2% normal pig serum for one hour at 37°C or overnight at 4°C). Bound antibody was detected by sequential application of swine anti-rabbit immunoglobulin (Dako Ltd; Z196; 1/30 for 30 minutes at room temperature), anti-rabbit peroxidase-anti-peroxidase (Dako Ltd; Z113; 1/100 for 30 minutes at room temperature), and diaminobenzidine substrate (BDH Ltd; 0.2 mg/ml in 50 mM Tris-HCl, pH 7.5, to which was added 6% H₂O₂ immediately before use). The reaction was stopped after five–10 minutes by washing with tap water.

Results

The feasibility of detecting HPV DNA by in situ hybridisation with biotinylated synthetic oligonucleotides was established using skin wart tissue from a hyperkeratotic hand wart and a simple hand wart. Both were strongly positive with HPV-1E6 oligonucleotide probes using Method 1 for the detection of bound probe.⁵ Detection Method 2 was found to be equally successful (table). Positive reactions were also obtained on tissues of vulvar and anal warts with HPV-6bE6 oligonucleotide probe using either detection method (table). These results reflected the DNA type observed with cloned probes, although the vulvar wart seemed to give more intense staining with the HPV-11 cloned probe and the anal wart with the HPV-6bE6 probe. Double staining to detect papilloma antigens and viral DNA was tried and it proved possible to distinguish the brown colour of antigen containing cells from the grey colour of DNA containing cells (fig 1). Antigen and viral DNA were found in all four tissues.

Of seven cervical tissues exhibiting varying degrees of dysplasia, HPV antigens were not detected in any. One (specimen 8, table), which had been positive with both HPV-16 cloned probe and HPV-16E6 oligoprobe in the previous study,⁵ was included. It gave positive reactions when tested with HPV-16E6 oligoprobe using either detection method. A further three, however, were negative with HPV-16E6 and detection Method 1, but gave clearly positive nuclear staining

Table In situ hybridisation in four skin lesions and seven cervical lesions using HPV synthetic oligonucleotide and cloned probes

		HPV-DNA probe								
		Cloned			Oligonucleotide			Oligonucleotide		
Specimen No	Histological appearance	HPV1 detection method 1	HPV11	HPV16	1E6 detection method 1	6bE6	16E6	1E6 detection method 2	6bE6	16E6
Skin lesions:										
1	Hyperkeratotic hand wart	+++	-	-	+++	-	-	+++	-	-
2	Simple plantar wart	+++	-	-	+++	-	-	+++	-	-
3	Vulvar warts, K +	-	++	-	-	+	-	-	+	-
4	Anal wart		(+)	-	-	++	-	-	++	-
Cervical biopsy specimens:										
5	No dysplasia, K -				-	-	-		-	-
6	No dysplasia, K -					-	-		-	+
7	CIN I, K +				-	-	-		-	-
8	CIN II, K +	-	-	+			+	-		+
9	CIN II, K +				-	-	-		(+)	+
10	CIN II, K +				-	-	-			+
11	CIN III, K -				-	-	-		-	-

K+/- = koilocytes present/absent;
 +++ = large numbers of nuclei containing HPV DNA;
 ++ = many patches of nuclei containing HPV DNA;
 + = occasional patches of positive nuclei;
 (+) = HPV containing nuclei only found with careful searching;
 — = no hybridisation.

Fig 1 Section of hyperkeratotic hand wart double stained for papilloma common antigen and papilloma DNA using HPV-1 E6 synthetic oligoprobe and anti-biotin IGSS detection. Antigen—brown; HPV-1 DNA—grey.

when detection Method 2 was used (table). Three of the four positive results were from patients with lesions of moderate dysplasia all showing koilocytosis, while the fourth (specimen 6) had shown colposcopic abnormalities that were not seen on histopathological examination. The positive reaction detectable in specimen 6 is shown in fig 2. None of the other cervical tissues examined stained with the HPV-6bE6 oligoprobe.

Discussion

Although Southern and dot blotting are often used^{8,9} rather than in situ hybridisation for HPV detection, the combination of morphological assessment with hybridisation on routinely processed sections has particular advantages in the diagnosis of HPV infection and its association with cervical intraepithelial neoplasia. Attempts to improve the sensitivity of bound biotinylated HPV probes by amplification with additional biotin-avidin layers² or by silver enhancement of horseradish peroxidase staining¹⁰ have been reported. Not only is the definition and localisation of DNA sequences better when obtained cytochemically,

but the time required for processing and the costs are also reduced. Few diagnostic virology or histology laboratories have the facilities to make probes from cloned nucleic acid, and the introduction of synthetically produced, defined probes, either species or type specific, depending on the sequence chosen, and available in large quantities at reasonable cost, provides an attractive alternative.

In the detection of hepatitis B virus DNA in serum, Lin *et al* reported using a radiolabelled oligonucleotide and found equal sensitivity in a shorter time than with larger nick-translated cloned probes.¹¹ Recently different strains of plant viroids were detected with specific radiolabelled oligoprobes, despite only a single nucleotide difference in their sequences.¹² Thus radiolabelled oligoprobes seem to offer at least equal sensitivity, greater specificity, and more rapid processing than conventional probes. While a comparison of radiolabelled and biotinylated oligonucleotides has not been made, differences in sensitivity can still be obtained with different detection systems, as shown in the current study.

The anti-biotin-IGSS detection system detected HPV-16 in four of seven cervical dysplasias; the

Fig 2 Section of cervical lesion showing hybridisation with HPV-16E6 synthetic oligoprobe and anti-biotin IGSS detection in specimen 6.

streptavidin-biotinylated enzyme system detected only one. Anti-biotin-IGSS has the added advantage of requiring less time for detection, particularly in the final development stages. Furthermore, by removing the streptavidin-amplification step, non-specific reactions due to endogenous biotin may be reduced. As it is now possible to biotinylate oligonucleotides at the time of synthesis, more consistent labelling should be achieved than with terminal transferase, and this may improve the sensitivity still further. While the role of HPV in cervical carcinogenesis is still in question,¹³ it is important to use the most sensitive and practical assay available to assess the biological importance of its presence in normal, dysplastic, and malignant lesions.

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References

- 1 Brigati DJ, Myerson D, Leary JJ, *et al*. Detection of viral genomes in cultured cells and paraffin embedded tissue sections using biotin-labelled hybridisation probes. *Virology* 1983;126:32-50.
- 2 Beckmann AM, Myerson D, Daling JR, Kiviat BN, Fenoglio CM, McDougall JK. Detection and localisation of human papillomavirus DNA in human genital condylomas by in situ hybridisation with biotinylated probes. *J Med Virol* 1985;16:265-73.
- 3 Forghani B, Dupuis KW, Schmidt NJ. Rapid detection of herpes simplex virus DNA in human brain tissue by in situ hybridisation. *J Clin Microbiol* 1985;22:656-8.
- 4 Ungar ER, Budgeon LR, Myerson D, Brigati DJ. Viral diagnosis by in situ hybridisation. *Am J Surg Pathol* 1986;10:1-8.
- 5 Cubie HA, Norval M. Synthetic oligonucleotide probes for the detection of human papilloma viruses by in situ hybridisation. *J Virol Methods* 1988;20:239-49.
- 6 Tourtellotte WN, Verity AN, Schmid P, Martinez S, Shapshak P. Covalent binding of formalin fixed paraffin embedded brain tissue sections to glass slides suitable for in situ hybridisation. *J Virol Methods* 1987;15:87-99.
- 7 Holgate CS, Jackson P, Cowen P, Bird CC. Immunogold silver staining—new method of immunostaining with enhanced sensitivity. *J Histochem Cytochem* 1983;31:938-44.
- 8 McCance DJ, Campion MJ, Chesters PM, Jenkins D, Singer A. Prevalence of human papillomavirus type 16 DNA sequences in cervical intraepithelial neoplasia and invasive carcinoma of the cervix. *Br J Obstet Gynaecol* 1985;92:1101-5.
- 9 Meanwell CA, Cox MF, Blackledge G, Maitland NJ. HPV-16 DNA in normal and malignant cervical epithelium: implications for the aetiology and behaviour of cervical neoplasia. *Lancet* 1987;i:703-7.
- 10 Burns J, Graham AK, Frank C, Fleming KA, Evans MF, McGee JO'D. Detection of low copy human papillomavirus DNA and mRNA in routine paraffin sections of cervix by non-isotopic in situ hybridisation. *J Clin Pathol* 1987;40:858-64.
- 11 Lin HJ, Wu P-C, Lai C-L. An oligonucleotide probe for the detection of hepatitis B DNA in serum. *J Virol Methods* 1987;15:139-49.
- 12 Sano T, Kudo H, Sugimoto T, Shikata E. Synthetic oligonucleotide hybridisation probes to diagnose hop stunt viroid strains and citrus exocortis viroid. *J Virol Methods* 1988;19:109-20.
- 13 Kitchener HC. Commentary: Does HPV cause cervical cancer? *Br J Obstet Gynaecol* 1988;95:1089-91.

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