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Detection and typing of human papillomavirus using the Vira Type "in situ" kit: comparison with a conventional dot blot technique

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Abstract

A new commercial kit (Vira Type "in situ", Life Technologies, Inc., Molecular Diagnostics Division, Guithersburg, Maryland, USA) for the detection of human papillomavirus (HPV) types 6, 11, 16, 18, 31, 33 and 35 in routinely processed human anogenital tissue was compared with a conventional dot blot assay for HPV 6, 11, 16 and 18. Both systems use double-stranded genomic DNA probes for the detection of type specific HPV DNA. The probes used on the dot blots were labelled with ^{32}P and visualised autoradiographically. The Vira Type probes were labelled with biotin and visualised using a streptavidin-alkaline phosphatase conjugate with NBT-BCIP substrate. Biopsy specimens from the cervix, vagina, and vulva of 46 women were processed by both methods and compared. The histological diagnoses ranged from benign changes, to dysplasia, and invasive carcinoma. Overall, 50% of biopsy specimens were positive for HPV DNA by dot blot hybridisation; only 39% were positive by Vira Type in situ hybridisation. Three of the specimens positive by the Vira Type "in situ" kit showed no cross hybridisation and were the same HPV type as the dot blot. A further 13 showed cross hybridisation, but the strongest Vira Type result corresponded to the dot blot results. One biopsy specimen was positive for different HPV types by the two tests and one was positive by Vira Type and negative by dot blot. Six biopsy specimens were negative by Vira Type but positive by dot blot.

It is concluded that the Vira Type "in situ" kit has a similar specificity but lower sensitivity than the dot blot hybridisation method for the detection of HPV DNA.

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Papillomaviruses are small, species specific DNA viruses which cause epithelial or fibroepithelial proliferations known as condylomas. Human papillomaviruses (HPV) have an icosahedral capsid and a covalently closed, circular, double-stranded DNA genome of about 8000 base pairs. HPV cannot be cultivated in vitro, and serological tests remain insensitive and relatively non-specific. The viral genome can be found throughout the thickness of an infected and condylomatous epithelium, but virion production and cytopathic effect are limited to the upper and terminally differentiated layers. Histology and immunohistochemistry for capsid antigens are, in practice, relatively insensitive. Moreover, HPV produce the same cytopathic effect regardless of type. Nucleic acid hybridisation techniques remain the most sensitive way of detecting infection, and the only reliable way of typing the HPV involved.[1 2]

Over 60 types of HPV have now been identified and they exhibit tropism for different epithelia. The anogenital area is consistently infected by types 6, 11, 16, 18, 31, 33 and 35, together with other less common types, such as HPV 42. It has become clear that types 6 and 11 are most often found in classic condylomata acuminata and subclinical "flat warts," or in low grade dysplasias. In contrast, high grade dysplastic lesions and invasive carcinomas are much more likely to be infected with HPV 16, 18, 31, 33 and 35. These HPV types can also be found in low grade lesions.[3] In condylomatous lesions HPV generally exist as free intranuclear episomes, but in genital carcinomas and some dysplasias they are integrated into the host cell genome.

Controversy still exists about the frequency with which low grade dysplasias progress to high grade dysplasia and to invasive carcinomas, but progression does occur in a proportion of patients.[4] Strong epidemiological and in vitro evidence suggests that lesions infected with HPV 16, 18, 31, 33 and 35 have a higher risk of progression to invasive carcinoma than types 6, 11.[5 6] A direct aetiological role for these HPV in malignant transformation, however, is yet to be proved. It has been suggested that the detection and typing of HPV in anogenital lesions may help to predict the clinical course of lesions, thereby allowing the allocation of screening and treatment programmes to be planned effectively.

Methods

The study group comprised 46 women referred between February 1987 and July 1988 to the Dysplasia Clinic of the Royal Women's Hospital, Melbourne, because of an abnormal cervical smear. Colposcopically directed target biopsy specimens were taken and bisected; failing that, adjacent target biopsy specimens were taken. One sample was sent for HPV DNA detection and the other for routine histological analysis.

HPV DNA DETECTION BY DOT BLOT HYBRIDISATION

Briefly, DNA was extracted with phenol-

chloroform after initial proteinase K digestion and then precipitated with ethanol.[7] Quantitation was performed by comparison with lambda phage (*Hind III*) DNA markers after gel electrophoresis. Duplicate samples of 3 μg of DNA were dotted on to nylon membranes (Zeta Probe, Bio Rad) using a 96-well vacuum manifold (Biodot apparatus, Bio Rad) and baked for 30 minutes at 80°C (instruction manual for DNA dot blotting, Bio-Rad). Genomic HPV DNA probes cloned into plasmid pBR322 (HPV 6, 11, and 16 into the *Bam H1* site and HPV 18 into the *Eco R1* site) were kindly supplied by Professor Harald zur Hausen, Heidelberg, West Germany. Plasmids were transformed into *Escherichia coli* HB 101 and then isolated by caesium chloride ethidium bromide gradient ultracentrifugation. HPV insert DNA was recovered using the appropriate restriction enzyme and purified by agarose gel electrophoresis. Inserts were labelled using ^{32}P dATP (Amersham UK) either by oligolabelling or by using the Multiprime DNA labelling kit (Amersham) to a specific activity of 2–6 × 10^8 cpm/μg of DNA.[8]

One of the pair of filters produced for each patient was hybridised with combined HPV 16/18 probes and the other with pBR322 DNA. Hybridisation was performed using 12–14 ng of radiolabelled probe/ml at high stringency (Tm −20°C) for 22 hours. Filters were washed once in 2 × SSC at room temperature for seven minutes, once in 2 × SSC (0·15M sodium chloride, 0·15M sodium citrate), 0·1% sodium dodecyl sulphate (SDS) at room temperature for 15 minutes and three times at 65°C for one hour in 0·1 × SSC and 0·1% SDS. The filters were left to autoradiograph for one to five days at −70°C with Kodak XAR-5 film and two intensifying screens. Both filters were then stripped of probe and one rehybridised with combined HPV 6/11 DNA and the other with pBR322 DNA, using the same conditions.

HPV DNA DETECTION USING VIRA TYPE KIT

Specimens sent for routine histological analysis were used for in situ hybridisation. Control sections, prepared from cell blocks of HeLa, CaSki, and SiHa cell lines, were also used. Tissue processing, pretreatments, hybridisation and detection were carried out according to the manufacturer's instructions with some minor modifications. Briefly, the procedure was as follows. Tissue was fixed in 10% buffered formol-saline for a variable period, paraffin wax embedded, and then cut at 4–5 μm. Seven sections per case were floated on to silanated slides, air dried and then baked for at least 30 minutes at 58°C before dewaxing through xylene and dehydrating in absolute ethanol. A protease/hydrochloric acid digestion step was followed by a TRIS-buffered saline wash and dehydration through graded ethanols. Forty to 80 microlitres of hybridisation buffer containing biotinylated HPV DNA probes were added to each of the sections as follows: the first was probed with combined HPV 6/11, the second with HPV 16/18, and the third with HPV 31/33/35. One positive and one negative control probe (human placental DNA and pBR322 DNA, respectively) were included each time and the remaining two sections were stained with haematoxylin and eosin for histological assessement.

Coverslips were carefully applied to exclude all air bubbles and the slides were then heated at 95°C for 15 minutes. Hybridisation was carried out for two hours at 37°C in a humidified chamber. Coverslips were removed by gentle agitation in TRIS-buffered saline with bovine serum albumin and three post-hybridisation washes performed using the same buffer at 37°C. Forty to 80 microlitres of streptavidin—alkaline phosphatase conjugate were added to the sections which were then incubated for 20 minutes at 37°C in a humidified chamber. The slides were washed with TRIS-buffered saline and then immersed in nitroblue tetrazolium chloride (NBT)—5-bromo-4-chloro 3-indolylphosphate p-toluidine salt (BCIP) substrate in TRIS-saline buffer for one hour at 37°C. The reaction was terminated by washing in distilled water and sections were then counterstained with nuclear Fast Red for 15 minutes. Sections were washed again with water, dehydrated through ethanols, cleared in xylene then mounted in DEPX (Gurr, BDH chemicals). The whole procedure, from sectioning to histological assessment, can be performed during a normal working day.

Results

Overall, 50% (23/46) of biopsy specimens were positive for HPV DNA by dot blot, with seven positive for HPV types 6 and 11 and 16 positive for HPV types 16 and 18. The detection limit for the dot blot assay was taken as 12·5 pg of HPV DNA. The correlation of HPV type with histology is given in table 1.

Vira Type "in situ" detected HPV DNA in 39% (18/46) of specimens, with six positive for types 6 and 11 and 12 positive for HPV types 16 and 18 (figs 1 and 2). The correlation with histology is shown in table 2. Twenty two biopsy specimens were negative by both assays. In three cases positive for HPV DNA the Vira Type and dot blot result agreed and there was no cross hybridisation with Vira Type results. A further 13 cases showed cross hybridisation with the Vira Type test (fig 2D). As suggested by the manufacturers, if a signal was evident in more than one probe group, the strongest signal was taken to represent the viral type, and in these 13 cases this agreed with the dot blot result. One biopsy specimen was positive for HPV DNA by both assays but for different types and one biopsy specimen was positive for HPV DNA by Vira Type "in situ" hybridisation but negative by dot blot. Six biopsy specimens were positive by dot blot but negative by Vira Type "in situ" hybridisation. The correlation between dot blot and Vira Type results is given in table 3.

Positive signals were obtained using Vira Type "in situ" for the CaSki and HeLa cell lines (500–600 copies of HPV 16 and 10–50 copies of HPV 18, respectively), but the signal in HeLa cells was very weak. No signal was obtained for the SiHa cell line (one copy of HPV 16).

Table 1 Correlation of HPV types by dot blot hybridisation with histological assessment of cervical biopsy specimens

	Histology						
HPV DNA type	*Normal*	*HPV infection*	*CIN I*	*CIN II*	*CIN III*	*Invasive squamous carcinoma*	*Total*
HPV 6/11	1	4	1	—	—	1-vulva	7
HPV 16/18	—	4	—	2	9	1-cervix	16
Negative	14	5	2	1	1	—	23
Totals	15	13	3	3	10	2	46

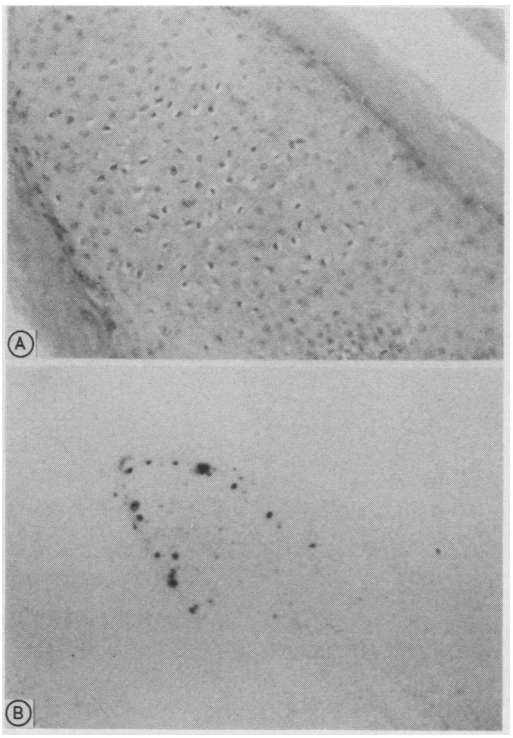

Figure 1 Photomicrograph of a typical vulval condyloma acuminatum. (A) Section showing a koilocytosis (haematoxylin and eosin). (B) Lower magnification showing a positive signal with a combined HPV 6/11 DNA probe: the signal is strongest in the upper layers of the epithelium (alkaline phosphatase–NBT–BCIP and nuclear Fast Red counterstain).

Discussion

Typing of papillomaviruses on routine anogenital biopsy specimens can be performed within one working day using the Vira Type "in situ" kit. While generally well designed and user-friendly, there was some scope for minor improvements to the kit. The manufacturers recommend that tissue be left in fixative for no more than 24 hours. Unfortunately this does not always occur in practice and may contribute to the lowered sensitivity of the kit relative to the dot blot method. Additionally, as emphasised by the manufacturers, sections cut on a dirty water bath increased the number of background hybridisation signals, especially from bacteria in the water. Heating sections on to silanated slides overnight at 60°C, instead of 30 minutes at 58°C, produced fewer lost sections and no appreciable reduction in signal strength.

It was suggested to mark around the section with a diamond pen to aid visualisation during the procedure, but this sometimes resulted in broken slides after denaturation at 100°C. An alcohol/xylene insoluble felt pen was found to be preferable. Although denaturing the section and probe DNA at 100°C on a heating block, several coverslips were forcibly dislodged by the hybridisation buffer boiling beneath them with obvious danger to the operator. Reducing the temperature from 100°C to 95°C in a hybridisation chamber and extending the time from five to 15 minutes avoided this danger and produced an adequate signal with improved morphological detail and fewer lost sections. The other small modification which was found to be useful was to increase the nuclear Fast Red counterstaining time from five to 15 minutes.

Signals were strongest in the upper layers of the epithelium and a strong signal provided no problems with interpretation. Some tissue sections, however, showed non-specific peripheral staining which could easily mask a weak signal. If more than one probe group produced a positive signal, the strongest signal was taken to represent the viral type and the others cross hybridisation. A true mixed infection, however, would be hard to interpret with this system. The hybridisation conditions used by the kit are effectively of medium stringency, and cross hybridisation between HPV groups is inevitable. An alternative strategy would be to screen at low stringency for all genital HPV to increase the sensitivity of the test, and then to type at high stringency to improve the specificity. This is already used by Life Technologies Inc. in their Vira-Pap Vira-Type kits for the detection and typing of HPV in cervical scrapes by dot blot.

The finding of two discrepant results could be a consequence of analysing different, albeit adjacent, specimens which harbour different HPV types. Additional HPV probes in the Vira

Table 2 Correlation of HPV type by Vira Type "in situ" hybridisation with histological assessment of cervical biopsy specimens

	Histology						
HPV DNA type	*Normal*	*HPV infection*	*CIN I*	*CIN II*	*CIN III*	*Invasive squamous carcinoma*	*Total*
HPV 6/11	—	4	1	—	—	1-vulva	6
HPV 16/18 (*HPV31/33/55)	—	2	—	3	7	—	12
Negative	15	7	2	—	3	1-cervix	28
Total	15	13	3	3	10	2	26

*All biopsy specimens showing a positive signal with the HPV 31/33/35 group were strongly positive for HPV 16/18 and the result read as HPV 16/18 positive with cross hybridisation.

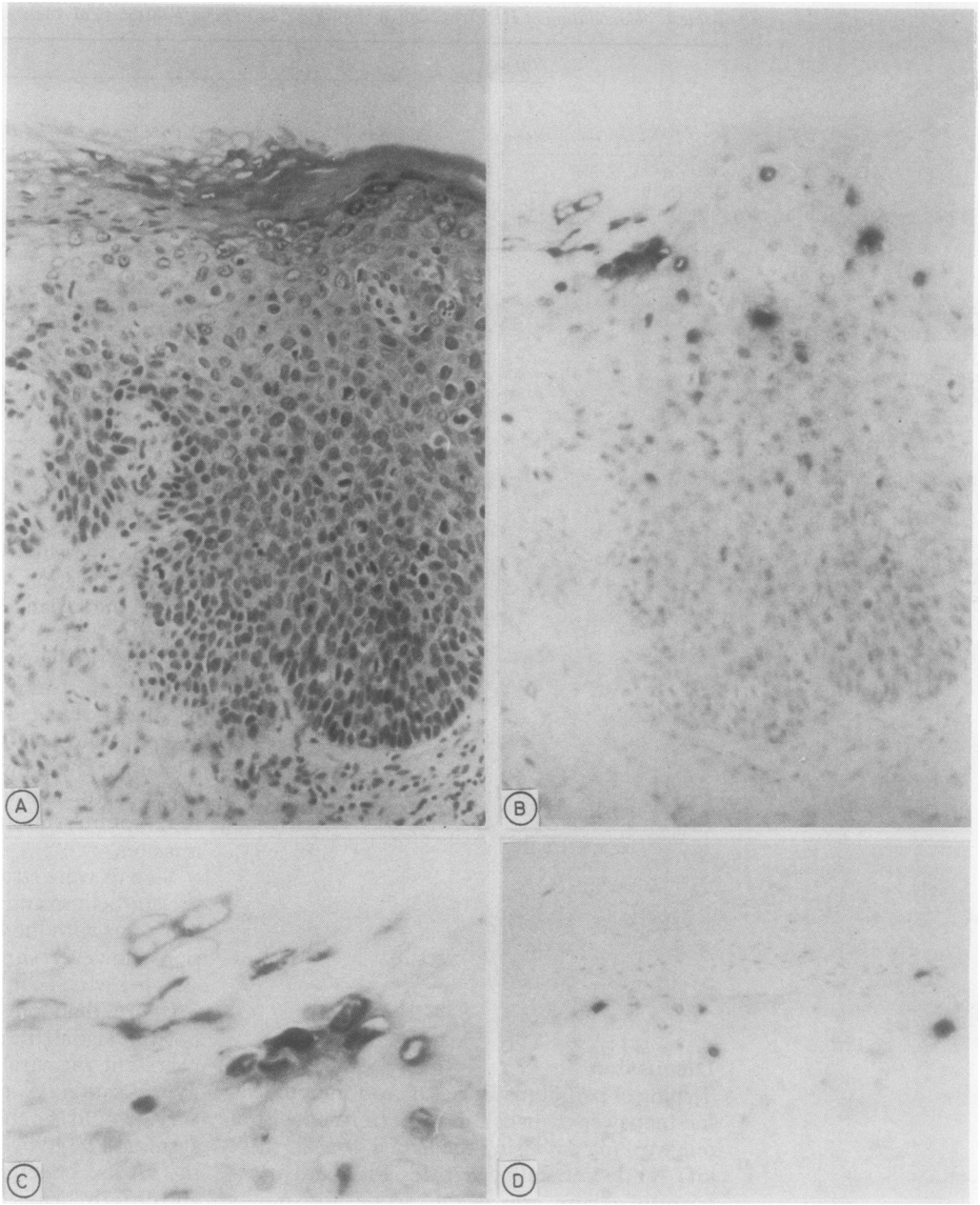

Figure 2 Photomicrograph of vulval intraepithelial neoplasia (VIN) grade III. This patient previously had a superficially invasive squamous carcinoma of the vulva. (A) VIN III (haematoxylin and eosin). (B) Same area as figure 2A showing a strong positive signal with a combined HPV 16/18 DNA probe. The signal is strongest in the upper layers of the epithelium, but positive nuclei can be seen towards the basal layer (alkaline phosphatase–NBT–BCIP). (C) High power view of figure 2B to show intranuclear localisation of HPV 16/18 DNA. (D) VIN III showing a weak positive signal with combined HPV 31/33/35 DNA probe. This weak signal was taken to represent cross hybridisation between HPV, most likely between HPV 16 and HPV 31 in this case. (This biopsy specimen was typed as HPV 16/18 positive).

Type kit (types 31, 33, and 35) and the use of medium stringency conditions could also permit cross hybridisation with other related HPV. These should not cross hybridise under the high stringency conditions of the dot blot. Dot blot hybridisation, although less specific and less sensitive than Southern Blot hybridisation, is much less labour intensive and permits relatively rapid screening of large numbers of clinical specimens. It still requires expensive equipment and specialised technical skills.

The Vira Type kit, although less sensitive than the dot blot assay, does have the advantage of not requiring special expertise or equipment. In addition, archival tissue can be used for retrospective studies and the lesion associated with the HPV infection can be assessed.

Currently, management of CIN depends on clinical and histological assessment of individual women and HPV typing is therefore largely of academic interest. If, however, the association between certain HPV types and

Table 3 Correlation of Vira Type "in situ" hybridisation result with dot blot result and histological assessment of cervical biopsy specimens

Vira type "in situ" hybridisation and dot blot results	*HPV DNA*	*Histology*						
		Normal	*HPV*	*CIN I*	*CIN II*	*CIN III*	*Invasive squamous carcinoma*	*Total*
Negative by both assays	Negative	14	5	2	—	1	—	22
Positive by both assays, no cross hybridisation	6/11	—	2	1	—	—	—	3
Positive by both assays, cross hybridisation	6/11	—	1	—	—	—	1-vulva	2
with Vira Type	16/18	—	2	—	2	7	—	11
Positive by both assays—	HPV 6/11 by Vira Type	—	1	—	—	—	—	1
different HPV types	HPV 16/18 by dot blot							
Negative by dot blot, positive by Vira Type HPV 16/18/31/33/35		—	—	—	1	—	—	1
Positive by dot blot,	HPV 6/11	1	1	—	—	—	—	2
negative by Vira Type	HPV 16/18	—	1	—	—	2	1-cervix	4
		15	13	3	3	10	2	46

carcinogenesis becomes more clearly established, HPV typing could have a role in the management of CIN. In order to assess this association more data from prospective follow up trials (with normal control groups) needs to be obtained, with analysis of HPV types, the lesions involved, and progression or regression rates. The Vira Type "in situ" kit would allow routine pathology laboratories to type HPV in biopsy material and provide some of these data.

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