

Easy Application of Digoxigenin-11-dUTP Labelled Probe in Detection of Human Papillomavirus DNA

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In situ hybridization was performed in ten cases of condyloma acuminata in order to study the applicability of digoxigenin-11 dUTP(Dig-dUTP) labelled probe compared with radioactive isotope labelled probes.

Although signal intensity was denser in radiolabelled probes, high positive rates were obtained with Dig-dUTP labelled probes. From these results, Dig-dUTP labelling is found to be more efficient in typing of human papillomavirus DNA than radiolabelling.

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Key Words : In situ hybridization, Condyloma acuminatum, Digoxigenin-11-dUTP, Radioactive isotope

Various methods of hybridization have been used to identify human papillomavirus(HVP). Among these techniques, in situ hybridization is directly applicable to the biopsy specimen and allows us to investigate the location of specific genes. Since this technique can be applied to the paraffin-embedded tissue, retrospective studies are also possible.^{1,2}

Radioactive isotopes including ³²P, ³⁵S, ³H have been used in hybridization experiments.³⁻⁵ Most isotopes have a short half life requiring frequent probe preparation. Safety issues and the high cost of radioactive waste disposal have stimulated the search for effective nonradioactive markers for the labelling of the probes.⁶⁻⁸

Recently digoxigenin was introduced as a new nonradioactive labelling material with good efficiency.⁹ In this study we applied Dig-dUTP labelled probes in condyloma acuminatum using in situ hybridization technique and compared the results with radiolabelled probes.

MATERIALS AND METHODS

Tissue Samples

Ten cases of condylomata accuminata formalin-fixed and embedded in paraffin were selected. For comparison of the different methods of in situ hybridization, three slides of each sample were prepared.

Pretreatment of Tissue Sections

Sections 4 to 6 μ m thick were placed on poly-L-lysine(Sigma, St. Louis) coated slides. They were deparaffinized in xylene two times and hydrated through graded alcohols to distilled

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water. Briefly, the slides were immersed in 0.2N HCl for 15 min., washed in 2X SSC, 5 μ EDTA solution, then digested by a solution of proteinase K.

Tissue blocking step was added when using isotope ^{35}S in addition to treatment of 0.1M tri-ethanolamine with 0.25% acetic anhydride to reduce background in cases of radiolabelled probes.

The slides were washed twice in PBS containing 0.2% glycine, then dehydrated through graded alcohols.

Detecting Probes

HPV 11 plasmid DNA was labelled with Dig-dUTP(Boehringer Mannheim, Mannheim, West Germany), ^{35}S -dCTP(Amersham, Amersham, U.K.) and ^{32}P -dCTP(Amersham, Amersham, U.K.) using a random primer method. Unlabelled nucleotides were removed on a Sephadex G-50 column(Sigma, St. Louis).

Prehybridization

Hybridization solution(5X SSC, 0.5% blocking solution(Boehringer Mannheim), 0.1% N-lauroyl sarcosine, and 0.02% SDS) was placed on the tissue section. The slides were incubated in a humidified box at 51°C for one hour.

In Situ Hybridization

Hybridization mixture(50 μ l) containing 5ng / μ l of a probe was pipetted onto the prepared section and the slides were covered with coverslips. They were placed on a hot plate and denatured for 2 min at 92°C and transferred to an incubator at 51°C in a humidified box for 18–42 hours.

After hybridization, the slides were immersed in 2X SSC, then coverslips were removed carefully. The slides were washed sequentially in 1X SSC at room temperature with agitation for 15 min., 0.1X SSC at 50°C two times. They were dehydrated through graded ethanols and allowed to air-dry.

Detection of Hybridization Signals

With Dig-dUTP probes, the slides were processed using a Nonradioactive DNA Labeling and Detection kit(Behringer Mannheim).

The slides were washed with buffer 1 for 1 min. They were drained of excess buffer and incubated with polyclonal sheep anti-digoxigenin, conjugated to alkaline phosphatase for 30 min. The slides were then incubated for enzyme catalyzed color reaction with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium salt and stained with eosin.

With radioactive probes, the slides were dipped in liquid photographic emulsion(Kodak Nuclear tract NTB2, Eastman Kodak Co.) and allowed to dry completely in a dark box. They were developed, fixed and stained with hematoxylin.

RESULTS

Positivity of the reaction

The results of the experiments are summarized in Table 1. There was 55.5% positive rate with Dig-dUTP labeled probes(Fig. 1). With radiolabelled probes, 12.5% and 30% positivity was achieved with ^{35}S and ^{32}P respectively (Fig. 2, Fig. 3).

Table 1. Positivity of Reactions

Probe	Total No.	Positivity(%)
^{35}S	8	1(12.5)
^{32}P	10	3(30.0)
Dig-dUTP	9	5(55.5)

Intensity of the signals

The signals were relatively intense with radiolabelled probes compared with Dig-dUTP probes(Table 2).

DISCUSSION

Among the hybridization techniques, in situ

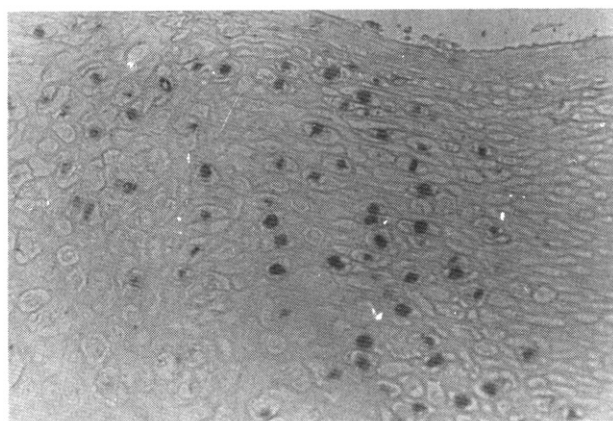


Fig. 1. With ^{325}S labeled probe, specific silver grains in the superficial layer of specimen ($\times 100$)

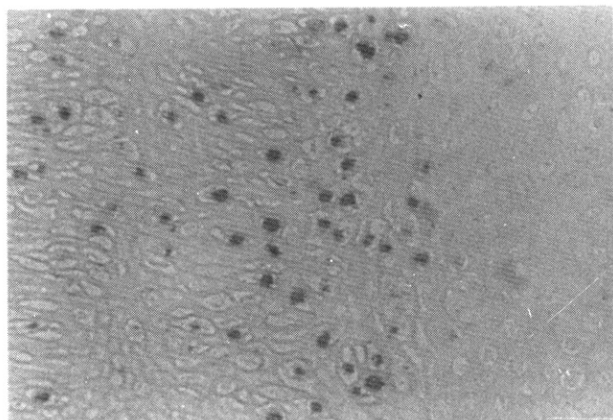


Fig. 2. Showing the signals using ^{32}P labeled probe ($\times 200$)

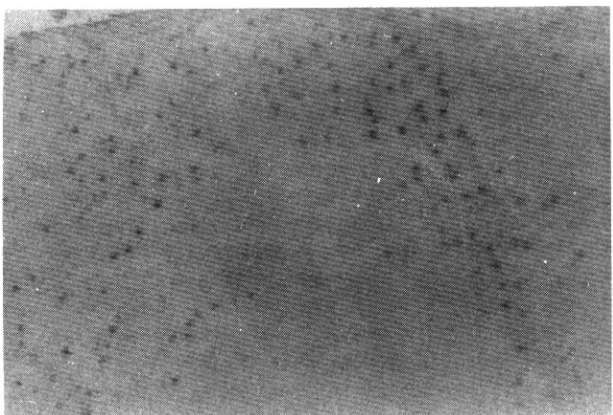


Fig. 3. Showing the signals using Dig-dUTP probe ($\times 100$)

Table 2. Intensity of Signals

Probe	Positive cases	Intensity		
		weak	moderate	intense
^{35}S	1			1
^{32}P	3		1	2
Dig-dUTP	5	1	3	1

hybridization is less sensitive than other hybridization techniques. This technique, however, permits study of the location of specific genes.

Generally, detection by nonradiolabelled probes is significantly less sensitive than radiolabeled probes.² Previous reports have given conflicting results of the sensitivity of these methods.^{3,7} Recently, a new nonautographic method using Dig-dUTP has been developed which is said to be very sensitive. Digoxigenin is a steroid which occurs naturally only in digitalis plants (*digitalis purpurea*). Its structure is shown in Fig. 4.

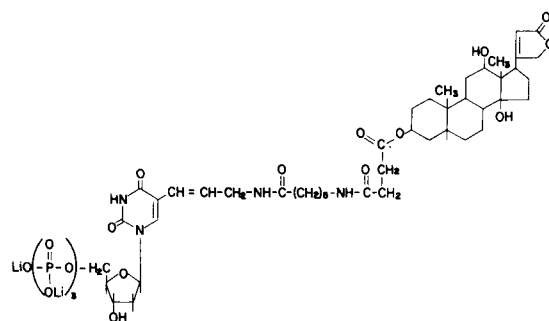


Fig. 4. Structure of Digoxigenin-11-dUTP

We tested Dig-dUTP labelled probes to compare the sensitivity of reactions using in situ hybridization. In previous reports, a 60–80% positive rate of detection was found in cases of condyloma accuminatum by in situ hybridization.^{1,11–13} In our experiments, we obtained positive rates of 55.5%, 30% and 12.5% with Dig-dUTP, ^{32}P and ^{35}S respectively. We believe the

low positive rates in our cases were caused less than optimally controlled conditions, especially in radiolabelled probes. If we controlled exposure time more adequately, we believe higher sensitivity rates would be obtained with the radiolabelled probes. Other possible factors include infections by HPV types different from 11 (eg. HPV 6 was three times as frequent as HPV 11) and a too vigorous washing process.

We also compared the signal intensity. Although only 12.5% and 30% of positivity were achieved by radiolabelled probes, three out of four reactions were intense in contrast with Dig-dUTP labelled probes which generated intense signals in only one out of five cases. Additionally there were more intense signals with radiolabelled probes than with Dig-dUTP labelled ones in three cases in which comparison of intensity was possible. These results show radiolabelled probes generated more intense signals with prolonged exposure time.

In this experiment, positive controls indicating adequate exposure time are necessary to increase positive rate of in situ hybridization using radiolabelled probes. Because HPV 6 and HPV 11 cannot be cultivated, there are no control samples to aid in detection, unlike HPV 16 or HPV 18 which are transfected into CaSki cell or Hela cell lines.

Compared with radiolabelled probes which take several days of exposure and dark room development, the enzymatic technique takes only a few hours, and is technically less difficult. In conclusion, Dig-dUTP labelled probes can be effectively used in the detection of human papillomavirus and other infectious agents. However, further studies are necessary to have reliable data on in situ hybridization using dig-dUTP probes.

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