DETECTION OF HPV DNA IN CLINICAL SPECIMENS BY ENHANCED CHEMILUMINESCENCE.

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SUMMARY

We have developed a sensitive method for detecting human papillomavirus (HPV) DNA in cervical scrapes using a non-isotopic, dot blot hybridization assay. For probe construction, the enzyme peroxidase is covalently linked to denatured DNA, through the amino group and the bases. Full-genomic insert HPV DNA is labeled in this way and used to screen HPV DNA in nucleic acid extracted from scraped cervical cells and spotted on nitrocellulose membranes. HPV DNA was detected in 46.9% of patients having cervical intraepithelial neoplasia (CIN) and in 21.4% of normal women. HPV 6/11 was the most prevalent type found in all patients. The method can detect from 1 to 10 pg of target DNA without background. The technique is easy to do, requires no special equipment and can be used in clinical settings.

RESUMEN

Se aplicó un método para la detección de ADN de *papillomavirus* humano (PVH) en raspados cervicales utilizando hibridación no radioactiva variante dot blot. Para la construcción de la sonda la enzima peroxidasa fue unida covalentemente al ADN desnaturalizado a través de los grupos amino y las bases de los ácidos nucléicos. El ADN del PVH fue detectado en un 46.9% de los pacientes que mostraban una neoplasia intraepitelial cervical (NIC) y en un 21.4% en mujeres normales, encontrándose una mayor prevalencia de PVH 6 11. El método fue capaz de detectar desde 1 hasta 10 pg de ADN, siendo la técnica de fácil realización ya que no requiere de equipamiento especial y puede ser utilizado en estudios clínicos.

INTRODUCTION

Human papillomavirus (HPV) belongs to a subgroup of the *Papovaviridae* family which are characterized as small, double-stranded, circular DNA viruses (Pfister, 1984). HPV genital tract infection may be one of the most common sexually transmitted diseases, being almost three times more frequent than genital herpes (CDC, 1986). Of the 16 types of HPV infecting the female genital tract, until now only a certain number are of clinical importance. The benign condylomata and low grade cervical dysplasia (CIN I) contains mostly HPV 6 and 11 whereas higher grade dysplasia (CIN II and III) and invasive cervical cancer usually contain HPV 16, 18 and 33 (Reid *et al.*, 1987).

The use of nucleic acid hybridization technique (Southern blot and dot blot) allows the detection and typing of HPV DNA. Both techniques are very sensitive and specific, but Southern blot is a laborious procedure, unsuitable for screening a large number of specimens (Roman and Fife, 1989). Because of its sensitivity, specificity and feasibility, dot blot is potentially suitable as a screening technique.

In this paper we applied a peroxidase labeled probe in a dot blot assay for screening cervical scrapes, looking for HPV DNA sequences.

MATERIALS AND METHODS

Population under study and preparation of samples

We studied 112 women presenting an abnormal cervical smear, that were under colcoscopy investigation in the Obstetric Maternity Hospital "Eusebio Hernández". A cervical scrape for routine cytology and a second scrape for the hybridization technique were taken with a wooden Ayre spatula. The cells collected in the second scrape were suspended in 6 ml phosphate buffered saline (PBS) with 0.1% sodium dodecyl sulfate (SDS). The sample was vigorously mixed to dislodge the cells from the spatula and the cells were counted in a haemocytometer. The number of cells obtained ranged from $2x10^4$ to $2x10^6$. The suspension was divided in two equal parts , both aliquots were pelleted by centrifugation and the pellets were stored at -20°C until required. A histopathological diagnosis was made according to the CIN Classification (Ferency, 1984), at the Pathology Department of the "Eusebio Hernández" Maternity Hospital.

Viral DNA probe

Cloned HPV6, 11, 16 and 18 were used during the studies. All four HPV DNA types were obtained as full length genomes, approximately 7.9 kb long; HPV 6, 11 and 16 were inserted into the BamHI site of pBR-322 (De Villiers *et al.*, 1981; Durst *et al.*, 1983; Gissman *et al.*, 1984) and HPV 18 into the Eco-RI site of pBR-322 (Boshart *et al.*, 1984).

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Cultures of bacteria and massive plasmid purification were performed as described (Maniatis *et al.,* 1982).

The purified plasmid was digested with the appropriate restriction endonuclease BamHI (Enzibiot, C. Habana, Cuba), and the viral inserts were purified by low gelling agarose techniques (Maniatis *et al.,* 1982). Control probe pBR-322 was prepared in the same manner.

Enhanced Chemiluminescence (ECL)

Viral probe was tagged with peroxidase which is covalently linked to denatured DNA, through the amino groups on the bases using an ECL gene detection Kit (Amersham, UK); based on the paper of Renz and Kurz (1984).

DNA extraction

DNA was extracted from the cells according to standard procedures. Cells were suspended in 1 mL of TE (10 mM Tris pH 0.1mM 7.4, EDTA pH 8): after digestion for 1 hour at 56°C with 250 μg/ml Proteinase K (Merk, Germany) in the pesence of 0.5% SDS, the samples were extracted twice with phenol:chloroform:isoamil alcohol (25:24:1) and DNA was precipitated with ethanol and suspended in 100 μl of TE buffer.

The concentration of DNA was calculated by gel electrophoresis and ethidium bromide coloration.

Dot Blot Hybridization

The DNA (1μg for each clinical samples) was denatured in 50 mM Tris-HCL ph 7.4; 0.2 N NaOH, 6XSSC (1xSSC: 0.15 M NaCL, 0.015 M Sodium citrate pH 6.8) for 10 minutes at 80°C. After neutralization with 0.2 M Tris-HCL pH 7.4, the samples were spotted onto nitrocellulose filters (Hybond ECL) by using a Manifold (Schleicher & Schuell, West Germany). The membranes were fixed for two hours at 80°C.

The hybridization and the detection reaction were carried out using the Amersham ECL gene detection Kit. For each probe 20 ng/ml of hybridization solution were used. Filters were hybridized sequentially; first with HPV types 6/11, then, with HPV types 16/18 and, due to the fact that in human genital samples the presence of pBR-322 is common (Bellomarino *et al.*, 1990), each filter was reprobed finally with pBR-322. The filters were exposed for autoradiography during one hour.

RESULTS AND DISCUSSION

DNA hybridization technique is the method of choice for detecting HPV in clinical samples, as the immunohistochemical detection of the virus is of limited value due to the lack of HPV type specific antisera. Usually, the probes have been labeled with ^{32}P, so, special precautions are required for handling radioactive materials, and this makes the assay unacceptable for routine clinical use. The purpose of the experiments reported here was to evaluate the ECL gene detection system as a non isotopic method of HPV detection for a possible clinical application in a group of patients.

In order to asses the sensitivity of the dot-blot hybridization, some strips of nitrocellulose were prepared containing DNA from the plasmid HPV16, the Hela and Siha cells as positive control; strips containing DNA from fibroblast, herpes simplex virus type 1 and 2 (HSV 1 and 2) and human cytomegalovirus (HCMV) were also prepared as a negative control (figure 1). All the strips were hybridized with the HPV 16/18 probe, washed and exposed one hour. This probe detected up to a level of 1 to 10 pg of HPV16 DNA; an amount of DNA corresponding to 104 Hela or 105 Siha cells was required to show a visible hybridization signal. Fibroblast, HSV 1 and 2, and HCMV controls remained negative. Only a low level of background was observed when more than 1 μg of fibroblast DNA were applied (data not shown).

HPV DNA in cervical samples

The patients included in the study ranged in age from 17 to 69 (mean=33.8) and each one had cytological results performed in a Referential Center. According to the results of the pathological diagnosis, patients were divided in two main categories: "normal", which mean normal cytology and histological features (n=4), and CIN patients (n=98), diagnosed according to the criteria of Ferency (1984).

HPV DNA was detected in 3 out of 14 patients without histological abnormality (21.4%) and in 46 of 98 patients having CIN diagnosis (46.9%) (see table 1). An example of such a blot is shown in figure 2. All samples were negative when the membranes were reprobed with pBR-322.

Table 1

Results for detection of HPV in clinical specimen by non isotopic DNA hybridization.

Cytology	N	6\11(%)	16\18(%)	all (%)	HPV +(%)
Normal	14	1(7.1)	1(7.1)	1(7.1)	3(21.4)
CIN I	39	9(23)	6(15.3)	3(7.6)	18(46.1)
CIN II	27	5(18.5)	3(11.1)	5(18.5)	13(48.1)
CIN III	32	6(18.7)	7(21.8)	2(6.2)	15(46.8)

The results of HPV typing were divided into three categories: a)patients being positive only to low risk types (HPV 6/11); b) patients being positive only to high risk types (HPV 16/18) and c) patients positive again both probes (HPV 6/11 and 16/18). Of the 46 patients with previous diagnosis of CIN being HPV DNA positive, 20 (43.4%) were of the low risk type, 16 (34.7%) were of the high risk type and 10 (21.7%) were positive by the two probes. For every grade of CIN, HPV 6/11

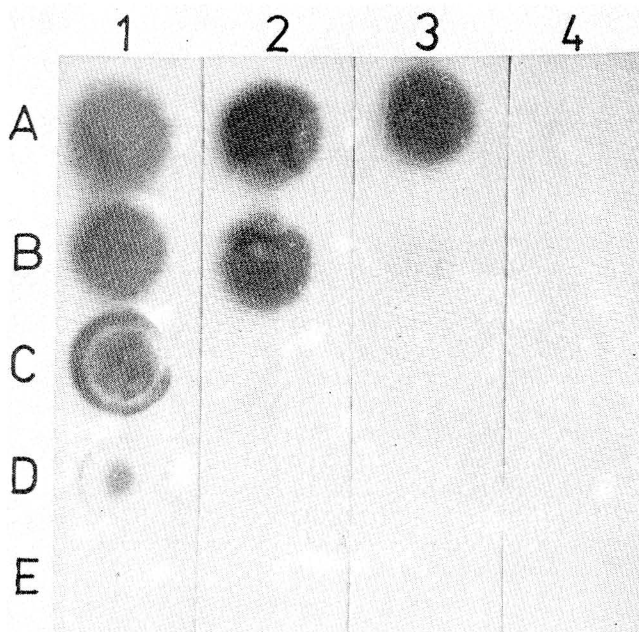

Fig. 1 Dot blot hybridization specificity and sensitivity. On strip 1, dilution of HPV 16 plasmid DNA ranged from 10 ng to 1 pg to 1 pg in 10-fold dilution. DNA extracted from 10^6, 10^5, 10^4, 10^3 and 10^2 Hela cells (strip 2) or Siha cells (strip 3) were mixed with 1 ug of salmon sperm DNA and applied on the filters. On strip 4 it was applied 1 μg of the following DNA: fibroblast (4A), Vero cells DNA (4B), HSV 1 (4C) and 2 (4D) and cytomegalovirus (4E). The four strips were hybridized with the HPV 16/18 probe washed and exposed as described. The probe could detect from 1 to 10 pg of the HPV 16 plasmid DNA, 10^4 Hela cells and 10^5 Siha cells. All the samples that were applied on strip 4 remained negative.

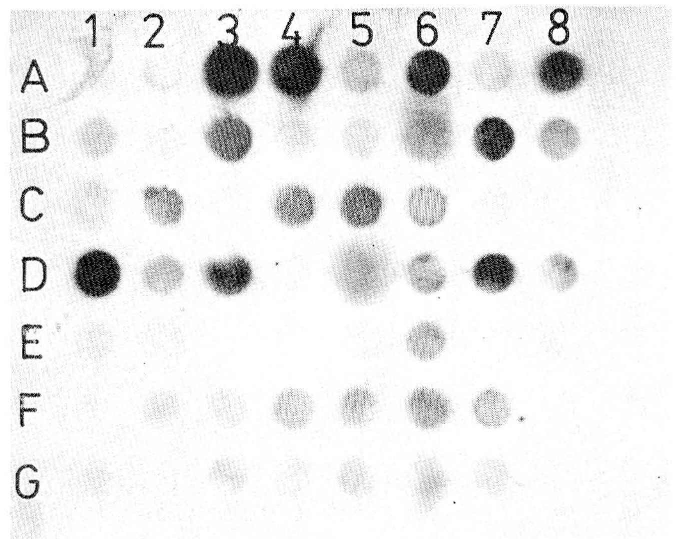

Fig. 2 Detection of HPV DNA in clinical samples by enhanced chemiluminiscence after one hour of autorradiography, the negative control, DNA extracted from Human fibroblastic cells, were located at positions A1 and A2, and positive control, DNA extracted from Hela cells, were located at positions A3 and A4; positive cases are considered only when the hybridization signal could be clearly differenciated from the one of the negative control.

was the most prevalent type. Positive reaction against both probes was seen more frequently in CIN grade II.

The failure of this method to identify HPV DNA in all CIN specimens may be due to the limited number of HPV probes used; only 4 probes were used, whereas more than 16 known HPV types have been identified in female genital tracts (Reid *et al.*,1987). The lesions proportion containing HPV 6/11 were similar to those reported by Reid *et al.*, (1987); also the prevalence of HPV DNA in normal patients is similar to the other previous reports (Campion *et al.*, 1986).

A number of methods have been described for non radioactive labelling of nucleic acid, ie: biotinylated nucleotide analog into double stranded DNA by nick translation (Hording *et al.*, 1983). The ECL system uses crosslinked probes consisting of single-stranded DNA and labeled protein moieties developed by Renz *et al.*, (1984), combined with enhanced chemiluminescence (Whitehead 1983). The specific advantage of this non-isotopic method is its safety. Moreover, labeled probes can be stored a -20°C in 50% of glycerol without significant lost of activity. Another advantage, specially useful in the case of HPV diagnosis, is that probes do not need to be removed before reprobing and filters can be rehybridized at least 6 times without a significant lost in sensitivity. In short, dot blot hybridization methods employing peroxidase labelling probes are potentially suitable as a routine HPV DNA screening technique.

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