

## Case report

# Detection of Epstein–Barr virus in a case of undifferentiated nasopharyngeal carcinoma by in situ hybridization with digoxigenin-labelled PCR-generated probes

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**Abstract.** A case of nasopharyngeal carcinoma is presented. Epstein–Barr viral genome was identified in the neoplastic cells by in situ hybridization with digoxigenin-labelled polymerase chain reaction-generated probes. We report the development of this technique in paraffin-embedded sections and propose that such identification may prove valuable for the diagnosis of this tumour in routine material.

**Key words:** Epstein–Barr virus – Nasopharyngeal neoplasms – In situ hybridization – Polymerase chain reaction

## Introduction

In 1921, Regaud and Schminke independently described a neoplasm occurring in the nasopharynx, characterized by anaplastic cells surrounded by many lymphoid cells (Regaud and Reverchon 1921; Schminke 1921). Light microscopic, electron microscopic and immunohistological studies of this neoplasm have confirmed its epithelial origin and the term “lymphoepithelioma”, initially given to this tumour, has been gradually replaced by the designation of “undifferentiated nasopharyngeal carcinoma with intense lymphoid stroma” or WHO type 3 nasopharyngeal carcinoma (UNPC; Shanmugaratnam and Sobin 1978).

The accurate identification of such tumours is difficult but important because of therapeutic and prognostic implications (MacComb 1972; Chu et al. 1984; Dickson et Flores 1985; Fedder and Gonzalez 1985). The nasopharynx may be hard to visualize and a primary lesion which tends to infiltrate submucosally is easily missed

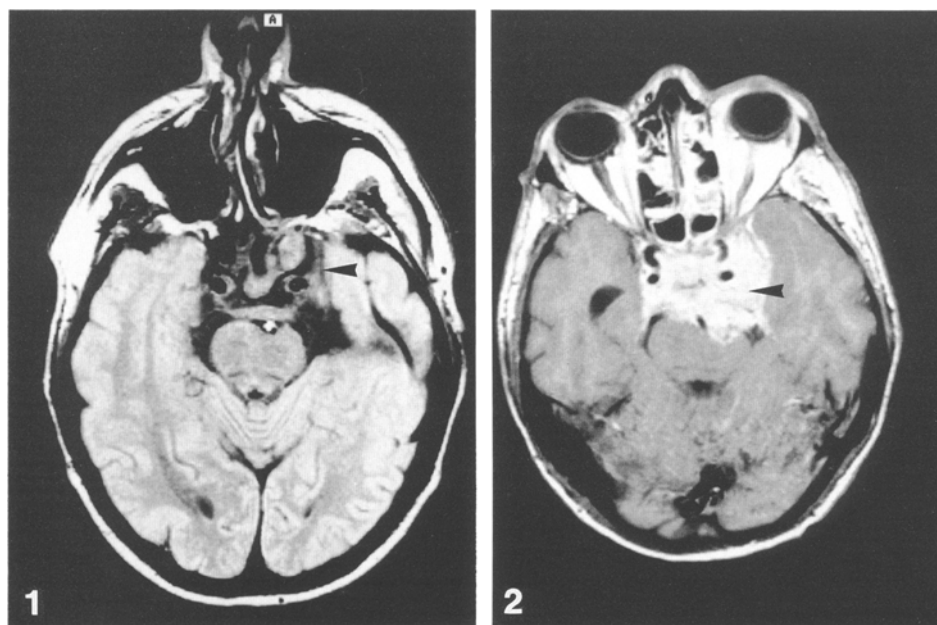
with superficial biopsies (Feinmesser et al. 1992a). Many tumours are detected late or remain undiagnosed until they present as metastases in the neck, often in the absence of evident pathological features at the primary site (Jesse et al. 1973). The pathological diagnosis may also be difficult because the epithelial origin of these undifferentiated or poorly differentiated tumours is not always clearly identifiable.

Epstein–Barr virus (EBV) has been associated with UNPC. This relation was first proposed in 1966 following the observation that serum from UNPC patients contained antibodies directed against lymphoblasts from productive, EBV-infected cell lines (Old et al. 1966). High titres of EBV-specific antibodies were subsequently demonstrated in UNPC patients from different parts of the world by immunofluorescence techniques (Henle et al. 1970; Desgranges et al. 1975; Henle and Henle 1976). The close link between EBV and UNPC was definitively established by the demonstration of EBV DNA by nucleic acid hybridization and polymerase chain reaction (PCR) (zur Hausen et al. 1970; Nonoyama et al. 1973; Akao et al. 1991; Feinmesser et al. 1992a). Viral-specific nuclear antigens were also identified in tumour epithelial cells but not in lymphoid cells by immunofluorescence (Huang et al. 1974; Chan et al. 1989). Since UNPC has been shown to be closely associated with EBV, easy methods for the identification of EBV might be useful for the diagnosis of this type of cancer, especially when the primary lesion is occult.

We report the case of a patient with an atypical clinical presentation of UNPC where the diagnosis was facilitated by the demonstration of EBV DNA in tumour cells by in situ hybridization. This technique, using digoxigenin-labelled PCR-amplified DNA fragments as probes on paraffin-embedded specimens, is described. Probes obtained by the direct incorporation of labelled nucleotides in the PCR reaction are also compared with a similar probe labelled after PCR by the random priming method.

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**Fig. 1.** Magnetic resonance imaging with gadolinium showing thickening of the left cavernous sinus (arrow) with little contrast enhancement

**Fig. 2.** Magnetic resonance imaging with gadolinium showing a very large lesion (arrow) invading the skull base, right and left cavernous sinuses, sella turcica and extending laterally towards the left temporal region and posteriorly to the brain stem. Contrast enhancement is very marked

## Case report

The patient was a 38-year-old man, who presented in November 1990 with a progressive diplopia. A few weeks later, he complained of left fronto-orbital headaches, left facial neuralgia and difficulties in swallowing. Neurological examination showed left abducens nerve palsy and left facial hypoaesthesia. Visual evoked potentials, cerebral CT scan and complete cerebral angiography were normal. Magnetic resonance imaging showed thickening of ethmoidal and sphenoidal sinus mucosa with contrast enhancement suggesting an inflammatory process; there was also a thickening of the lateral wall of the left cavernous sinus extending to the clivus (Fig. 1). A diagnosis of cavernous sinus meningioma was proposed and no therapeutic intervention was made.

The patient was referred in February 1992 with bilateral abducens nerve palsy, complete loss of vision in the left eye, left facial neuralgia, left facial hemihypoaesthesia, a nasal voice, accentuation of the swallowing trouble and a soft palate palsy. He had lost 15 kg in weight during the previous year and had an enlarged left cervical lymph node. Magnetic resonance imaging at this time showed an extensive tumour of the skull base, invading the sella turcica, the left cavernous sinus and temporal fossa, the sphenoidal and ethmoidal sinuses, reaching the right cavernous sinus and eroding the clivus (Fig. 2). As the tumour was extending to the cavum, a biopsy of the lesion was realized by a transnasal approach. Histological examination showed an undifferentiated tumour with an intense lymphoid stroma. The diagnosis of UNPC was confirmed by the demonstration of EBV DNA in neoplastic cells by in situ hybridization.

Serology performed at the time of diagnosis revealed high titres of EBV-specific antibodies. Treatment combining chemotherapy and radiotherapy was carried out.

## Materials and methods

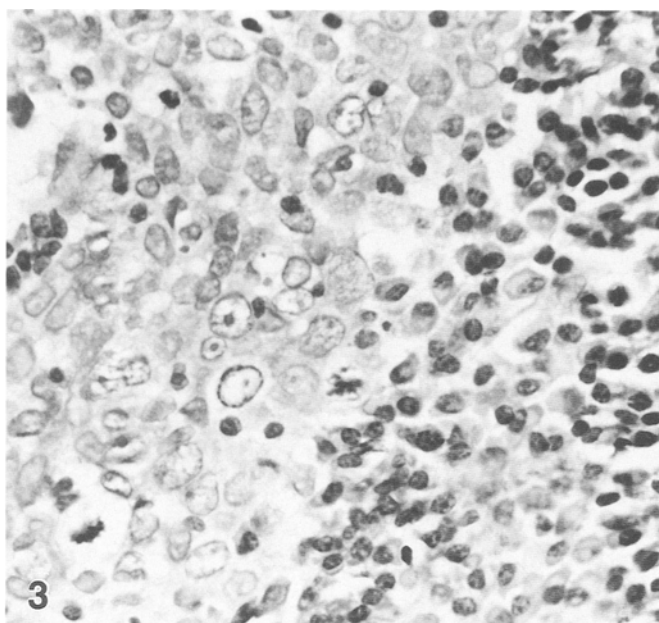
The production of digoxigenin-labelled probes by PCR was carried out as follows: a 110 base pairs (bp) fragment of EBV DNA was amplified by PCR with primers hybridizing in the *Bam*H1 W fragment of EBV (Table 1; Cheung and Kieff 1982; Baer et al. 1984; Saito et al. 1989) in a DNA preparation of a B lymphoid cell line immortalized by EBV (LHN13) in the presence (or absence) of digoxigenin-dUTP (Dig-dUTP; Boehringer Mannheim) in the reaction mixture. Different percentages of dTTP substituted by Dig-

**Table 1.** Sequence of Epstein-Barr virus (EBV) primers (Saito et al. 1989)

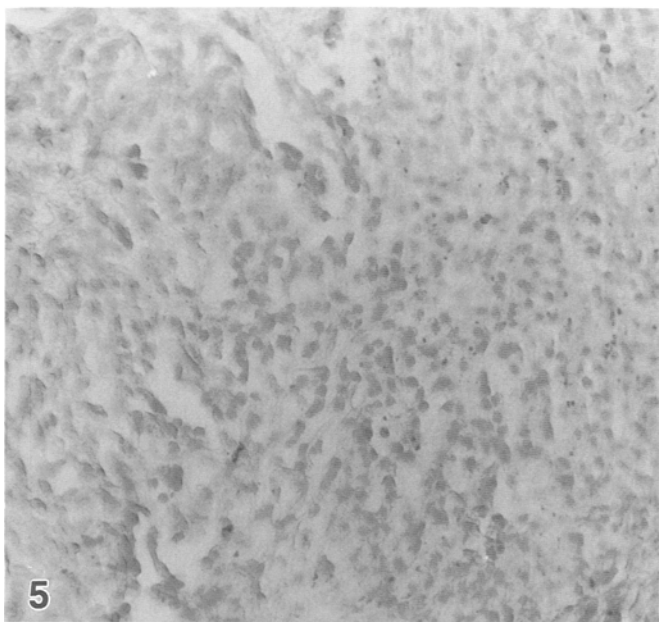
Region	Sequence	Location in genome of EBV
<i>Bam</i> W		
TC60 Primer	CCAGAGGTAAGTGGACTT	1396-1416
TC61 Primer	GACCGGTGCCTTCTTAGG	1520-1503

dUTP in the PCR amplification solution were investigated (25%, 50%, 75% and 100%). PCR was carried out using the Perkin Elmer Cetus DNA thermal cycler with the following protocol: Hot-Start method at the initiation of cycling (Erlich et al. 1991); annealing, 55° C for 2 min; extension, 72° C for 3 min; denaturation, 94° C for 1 min; for 30 cycles. The 50 µl PCR mixture contained about 1 ng of previously amplified target DNA, 10 mM-TRIS hydrochloric acid (TRIS-HCl; pH 8.3), 50 mM potassium chloride, 1.5 mM magnesium chloride ( $MgCl_2$ ), 1 µM of each primer, 200 µM of each dATP, dCTP, dGTP, 1.25 U *Taq* DNA polymerase (Promega) and different ratios of Dig-dUTP: dTTP: 0 µM Dig-dUTP: 200 µM dTTP (0% substitution with Dig-dUTP), 50 µM Dig-dUTP: 150 µM dTTP (25% substitution with Dig-dUTP), 100 µM Dig-dUTP: 100 µM dTTP (50% substitution with Dig-dUTP), 150 µM Dig-dUTP: 50 µM dTTP (75% substitution with Dig-dUTP), 200 µM Dig-dUTP: 0 µM dTTP (100% substitution with Dig-dUTP). After amplification and ethanol precipitation, the probes were dissolved in 100 µl TE pH 8 (10 mM-TRIS-HCl pH 8.0-1 mM EDTA pH 8.0). The DNA sample produced by PCR without incorporation of Dig-dUTP in the reaction mixture (0% substitution with Dig-dUTP) was labelled in vitro by random priming (Feinberg and Vogelstein 1983) with Dig-dUTP to be used as probe.

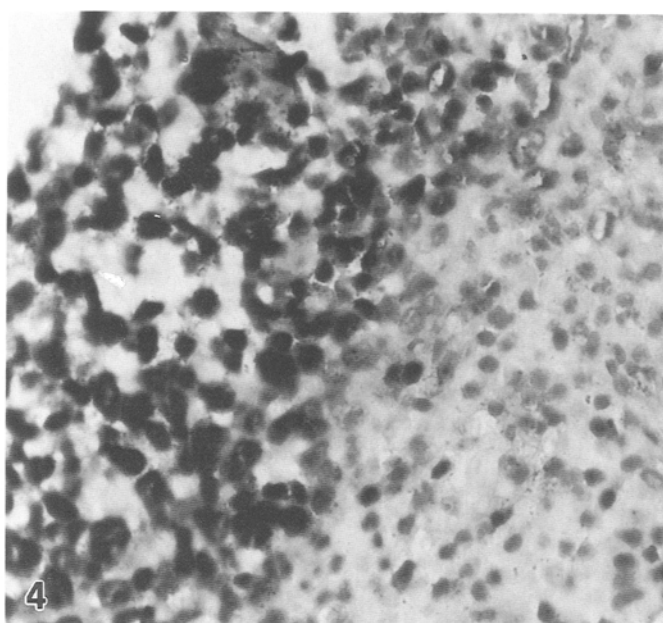
In situ hybridization was performed as previously described for human papillomavirus (Nuovo and Richart 1989; Delvenne et al. 1992) with several modifications. Briefly, 4-µm tissue sections were placed on slides pretreated with a 2% solution of organosilane (Aldrich, Milwaukee, Wis.) in acetone. The slides were deparaffinized for 5 min in xylene, dehydrated in alcohol and treated for 10 min with a solution containing 100 µg/ml proteinase K (Boehringer Mannheim). The hybridization mixtures were made with 500 ng/ml digoxigenin-labelled probes in 50% deionized formamide,



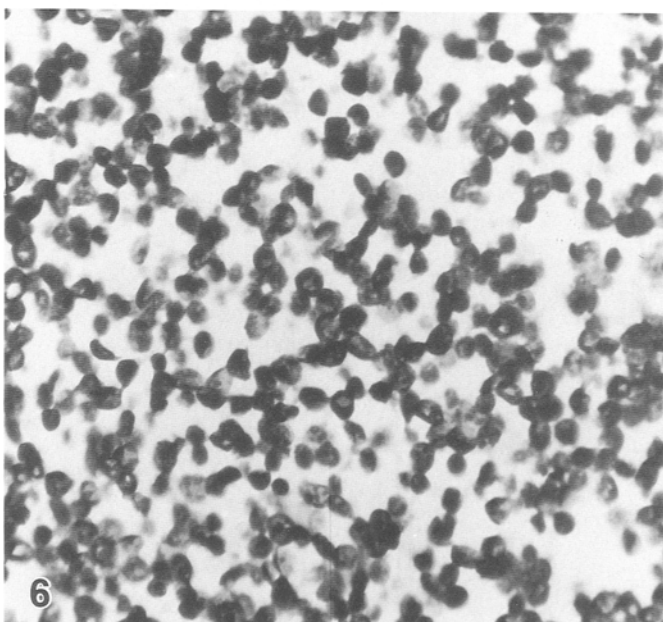
**Fig. 3.** The tumour is composed of ill-defined nests of carcinoma cells with an abundant lymphoid infiltrate (Regaud pattern). Haematoxylin and eosin,  $\times 250$



**Fig. 4.** Detection of Epstein-Barr virus in nasopharyngeal carcinoma by in situ hybridization with a polymerase chain reaction probe labelled with digoxigenin-dUTP by the random priming method. Hybridization signal is confined to nests of large malignant epithelial cells. Small surrounding lymphocytes and stromal cells show counterstain only.  $\times 250$



**Fig. 5.** The negative control using a cytomegalovirus digoxigenin-labelled probe on the same block of tissue as that shown in Fig. 4 contains virtually no background staining.  $\times 100$



**Fig. 6.** Positive control using a polymerase chain reaction probe labelled by the direct incorporation of digoxigenin-dUTP in the amplification reaction on a section of a formalin-fixed paraffin-embedded LHN13 cell pellet. Most cells are positive and background staining is absent.  $\times 250$

10% dextran sulphate and  $3 \times \text{SSC}$  ( $20 \times \text{SSC}$ : 3.0 M sodium chloride (NaCl), 0.3 M sodium citrate). After denaturing the probe mixtures and tissues together at  $100^\circ \text{C}$  for 5 min, the hybridization reaction went for 12 h in a humid chamber at  $37^\circ \text{C}$  beneath plastic coverslips (Clavies, Bel-Art Products, Pequannock, N. J.). We found several advantages in using these plastic coverslips: the lower cost of the test because the hybridization solution volumes necessary to cover the tissue were decreased and there was no desiccation of the tissue during the denaturation and hybridization steps, even

in the absence of sealing hybridization chambers. Unhybridized probes were removed by washing the slides in 2% bovine serum albumin in  $0.2 \times \text{SSC}$  for 10 min at room temperature. The slides were incubated at  $37^\circ \text{C}$  for 30 min with an alkaline-phosphatase-conjugated anti-digoxigenin antibody (Boehringer Mannheim) diluted 500 times in 0.1 M TRIS-HCl, pH 7.5, 0.15 M NaCl. After rinsing off excess antibody with TRIS-saline (0.1M TRIS-HCl pH 9.5; 0.1M NaCl; 50 mM  $\text{MgCl}_2$ ), the slides were incubated for 60 min at  $37^\circ \text{C}$  in the presence of alkaline phosphatase substrates

nitroblue tetrazolium and bromo-chloro-indolylphosphate. This alkaline phosphatase-based colorimetric detection method resulted in a purple-blue precipitate at the site of hybridization. Finally, the sections were counterstained with nuclear fast red, dehydrated, cleared and mounted in permanent mounting medium. The specificity of the system was confirmed by using digoxigenin-labelled probe made from sequences homologous to cytomegalovirus. LHN13 EBV-positive cells were used as positive controls.

## Results

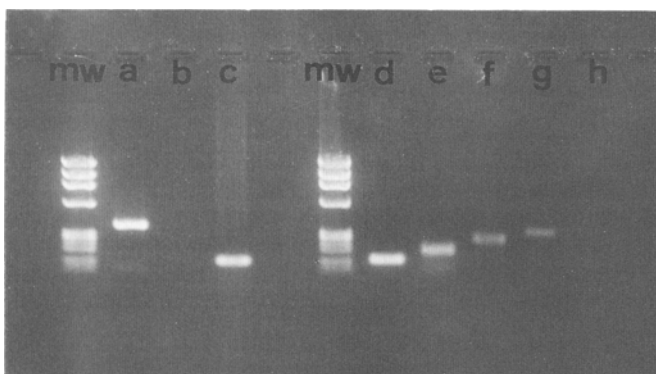
The predominant pathological finding was the presence of sheets of anaplastic cells surrounded by an abundant lymphoid infiltrate (Fig. 3). An immunoperoxidase study for keratin (KL1 Immunotech) labelled the neoplastic cells, suggesting the diagnosis of UNPC. This diagnosis was confirmed by the demonstration of EBV DNA in malignant epithelial cells but not in lymphoid cells by *in situ* hybridization with probes labelled by the random priming method or the direct incorporation of Dig-dUTP in the PCR (Fig. 4). No hybridization signal was seen in areas of apparently normal epithelium. Negative and positive controls gave the expected results (Figs. 5, 6). Amplified EBV DNA was also detected by PCR in the crude DNA solution obtained from serial sections of the tissue by deparaffinization and boiling as described previously (Nuovo et al. 1991; Fig. 7). The efficiency of amplification in the presence of digoxigenin-labelled nucleotides was evaluated by agarose gel analysis of the PCR products. We obtained amplification of DNA with percentages of dTTP substituted by Dig-dUTP of 25%, 50% and 75%. When the proportion of Dig-dUTP was increased to 100%, no amplification was observed. Incorporation

of labelled nucleotides resulted in a slight increase in the molecular weight of amplified products, causing slightly decreased electrophoretic migration (Lo et al. 1988). A higher ratio of Dig-dUTP:dTTP produced slower mobility (Fig. 7). The incorporation of Dig-dUTP into the PCR product was also confirmed by Southern transfer of the PCR products onto nylon membrane and detection with alkaline-phosphatase-labelled anti-digoxigenin antibody (data not shown). Comparison of the *in situ* hybridization results with the various digoxigenin-labelled probes did not show major differences of signal or background intensities.

## Discussion

The infiltrative characteristics of nasopharyngeal carcinoma and its submucosal extension often make this type of cancer difficult to diagnose (Feinmesser et al. 1992a). In many patients the first evidence of disease is cervical lymph node metastases, often with little overt evidence of disease at the primary site (Jesse et al. 1973). In our case report, the tumour was only diagnosed after skull base invasion with cranial nerve palsy and after having first been mistaken for a meningioma of the cavernous sinus, so that more than 1 year elapsed between the onset of first symptoms and diagnosis.

Therapeutic decisions for patients with primary tumours at unknown sites are generally based on clinical and pathological data. Since the epithelial origin of UNPC is not always clearly identifiable and since EBV has been shown to be a reliable marker for these tumours (Feinmesser et al. 1992a), there is a need for a relatively simple and rapid technique which is capable of identifying EBV in these patients. Although the presence of antibodies to EBV in serum is very common in patients with UNPC, such antibodies are also found in the general population. Several studies have reported that serum levels of anti-EBV antibodies are not reliable indicators of occult nasopharyngeal tumours (Cai et al. 1983; Pearson et al. 1983). Other techniques to detect EBV infection rely on the use of molecular probes to identify EBV genomes in tissue extracts by Southern blotting or by PCR (Peiper et al. 1990; Coates et al. 1991a). Southern blotting is usually performed with radioactive probes which require special handling and disposal of radioisotopes. Nonisotopic filter hybridization techniques are more recent and rapid methods but they do not circumvent the problem of using fresh or frozen tissue for DNA extraction. Moreover, it is sometimes difficult to obtain enough tissue for Southern blot analysis, especially in UNPC since surgery is rarely indicated. PCR is sensitive and relatively easy to perform but does not provide any information about the type, the proportion and the histological localization of cells containing viral nucleic acids within tissue sections. In contrast, the technique of *in situ* hybridization allows the detection of viral specific DNA or RNA sequences within cells compatible with microscopic examination and requires only a small amount of material. In our study, we have made use of a 110 bp DNA sequence amplified in the



**Fig. 7.** Agarose gel analysis of polymerase chain reaction (PCR) products visualized by UV light after ethidium-bromide staining. Amplified Epstein-Barr virus (EBV) DNA was detected by PCR at the expected size (110 bp) in the DNA solution obtained from serial sections of the undifferentiated nasopharyngeal carcinoma biopsy specimen (lane c). Primers amplifying a 370 bp fragment in a human genomic sequence (MP6d-9: single copy gene near the cystic fibrosis locus on human chromosome 7; Huth et al. 1989) were also used to rule out inability to amplify DNA (lane a). DNA from a renal biopsy tested with EBV primers was used as negative control (lane b). Lanes MW contained DNA molecular weight standards ( $\Phi \times 174$ , BRL). The effect on the PCR products of incorporation of different percentages of digoxigenin-dUTP in the reaction mixture is also shown: 0% (lane d), 25% (lane e), 50% (lane f), 75% (lane g) and 100% (lane h).

*Bam*H1 W fragment of EBV which is reiterated 7–12 times in different viral isolates (Cheung and Kieff 1982; Baer et al. 1984; Saito et al. 1989). For maximal sensitivity, this DNA sequence was labelled with digoxigenin and used as probe. We did not observe differences in the sensitivity of the probes labelled by the random priming method or by the direct incorporation of optimal amounts of Dig-dUTP in the PCR reaction (25–75% of dTTP substituted by Dig-dUTP). These results are confirmed by other authors who have shown the same DNA detection level for parvovirus in dot and Southern blot experiments with probes labelled by these two methods (Yun and Hornsleth 1991). The digoxigenin-based detection system has been shown to be more sensitive in detecting human papillomavirus DNA in tissues when compared with biotinylated probes (Furuta et al. 1990; Morris et al. 1990; Delvenne et al. in preparation) and to be able to detect the EBV genome with a sensitivity, in terms of DNA sequence, as low as 22–36 kbp (Coates et al. 1991b). Several in situ hybridization techniques with different types of probes (cDNA, RNA and oligonucleotide probes) and different detection systems have also been shown to be reliable for the detection of EBV DNA in routinely processed UNPC specimens (Coates et al. 1991b; Wu et al. 1991; Brousset et al. 1992). Some commercially available probes appear to work as well (Hawkins et al. 1990; Akao et al. 1991; Samoszuk 1991; Feinmesser et al. 1992b). We found several advantages in using a PCR-amplified DNA fragment as probe. Large amounts of DNA can be synthesized rapidly without any further purification. The PCR products are usually the optimal size to be used as probes for in situ hybridization (100–1000 nucleotides). Specific probes can be generated from a previously positive specimen in the absence of plasmids or any cloned DNA fragment if at least a part of the DNA sequence to amplify is known for the synthesis of primers.

In conclusion, the procedure described in this report permitted the reliable identification of EBV in routinely prepared tissue material and was useful in making the diagnosis of nasopharyngeal carcinoma in our patient. This method may be helpful in detecting the presence of viruses in a wide variety of tissues to investigate proposed associations with neoplastic diseases and sites of viral latency.

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