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High sensitivity detection of JC-virus DNA in postmortem brain tissue by *in situ* PCR

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Opportunistic infection of the central nervous system by human polyomavirus JC can cause a devastating disease, progressive multifocal leukoencephalopathy (PML). To gain new neuropathological insights into JC-virus (JCV) infection patterns in PML at the light microscopic level, the highly sensitive indirect *in situ* polymerase chain reaction (*in situ* PCR) was employed in up to 15-year old formalin-fixed and paraffin-embedded postmortem brain tissue derived from nine AIDS patients with PML. *In situ* PCR, in which target DNA is amplified intracellularly and detected by a specific labelled probe in morphologically intact tissue, was compared with conventional *in situ* hybridization (ISH). Validity was ensured by the inclusion of 13 controls. JCV detection with *in situ* PCR proved to be highly sensitive since in all nine brain samples the number of positive cells exceeded the ISH results by 2 – 3-fold. Whereas by routine staining the brain tissue of each individual patient showed regions with severe, mild or no involvement by PML, improved detection of JCV DNA by *in situ* PCR allowed a regrading into five different degrees of JCV infection. Significant myelin staining was observed, suggesting that cell-to-cell contact may not be the only means of virus spread but that new cells could also be infected by virus released after cell lysis. Furthermore, using *in situ* PCR hitherto unreported intracellular distribution patterns of JCV DNA in oligodendro- and astrocytes were observed by light microscopy. *Journal of NeuroVirology* (2000) **6,** 61 – 74.

Keywords: *in situ* PCR; *in situ* hybridization (ISH); progressive multifocal leukoencephalopathy (PML); JC-virus (JCV); human immunodeficiency virus (HIV)

Introduction

Progressive multifocal leukoencephalopathy (PML) is a subacute demyelinating disease of the central nervous system (CNS). It was first recognized in the late 1950s (Aström *et al*, 1958) and was relatively uncommon before the epidemic of acquired immunodeficiency syndrome (AIDS). PML may occur in human immunodeficiency virus- (HIV^-) infected patients when immune integrity is severely impaired by depletion of $CD4^+$-cells under a crucial threshold of 150 per mm^3 (von Einsiedel *et al*, 1993). Human polyomavirus JC (JCV) is the causative agent of PML and primarily infects the myelin-producing oligodendrocytes with subsequent cell lysis, demyelination and pathognomonic light microscopic features on routine tissue staining of affected brain (Richardson, 1961). Because JCV DNA is present in high copy numbers within oligodendrocytes, specific diagnosis is possible by conventional *in situ* hybridization (ISH) (Aksamit *et al*, 1986, 1990; Mori *et al*, 1991; Einsiedel von *et al*, 1993), for which at least 200 to 1000 copies of viral genome per cell are required (Houff *et al*, 1988).

Despite extensive investigation (Ferrante *et al*, 1995; Monaco *et al*, 1996; Agostini *et al*, 1997a,b; Antinori *et al*, 1997; Ault, 1997; Dubois *et al*, 1997; Gallia *et al*, 1997; Jochum *et al*, 1997; Kitamura *et al*, 1997; Weber *et al*, 1997; Weber and Major, 1997; Lafon *et al*, 1998) the pathogenesis of PML can only partly be resolved with available techniques. The

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highly sensitive *in situ* polymerase chain reaction (PCR) method promises to provide advantages for the investigation of PML. Theoretically, one JCV genome copy per cell can be detected with this combined molecular biological method (Nuovo *et al*, 1991a, 1993; Bagasra *et al*, 1993). An indirect approach with sequence-specific detection of the amplified DNA was utilized here because the signals are more specific than with direct *in situ* PCR, for which labelled nucleotides are incorporated into the amplicons during thermal cycling (Long *et al*, 1993; Sällström *et al*, 1993; Komminoth *et al*, 1994b; Ohshima *et al*, 1995). Formalin-fixed and paraffin-embedded postmortem tissue is a highly problematic sample material for *in situ* PCR requiring careful validation of the method.

This is a postmortem study of AIDS patients whose death was directly attributable to PML. Our aim was the microscopic evaluation of the intracerebral progression of PML, more specifically the determination of the number of JCV infected cells as well as of the amount and location of the viral DNA in brain tissue. For this purpose tissue chosen from different regions with absent, mild or severe involvement by PML as judged by routine staining (Figure 1) was investigated with two molecular biological techniques and the results were compared.

Results

Improved detection of JCV DNA by in situ *PCR*
In situ PCR detected viral DNA in all brain tissues from nine PML patients with a higher sensitivity than by ISH. The results of the technique were reproducible, and the interobserver variation was less than 5%. Generally, the number of *in situ* PCR-positive cells always exceeded that of ISH-positive cells by 2–3-fold. Cell counts per area from parallel tissue sections are compared in Figure 2. On a single cell level the hybridization signal appeared to be more intense after amplification than without, indicating that more templates were present after *in situ* PCR (Figure 3a–d). The morphology of the tissue, i.e. cells and the neuropil, remained intact. It is remarkable that in some necrotic regions no JCV-infected cells were detected by ISH but up to 90 cells/mm^2 were revealed by *in situ* PCR (Figures 2 and 5). After amplification by *in situ* PCR there were some cases with strikingly more viral DNA in the neuropil than on parallel ISH sections (Figure 3a–d).

Distribution of JCV DNA in tissues and within oligodendrocytes and astrocytes
As expected, regions of infection were most often located at the corticomedullary junction or in the

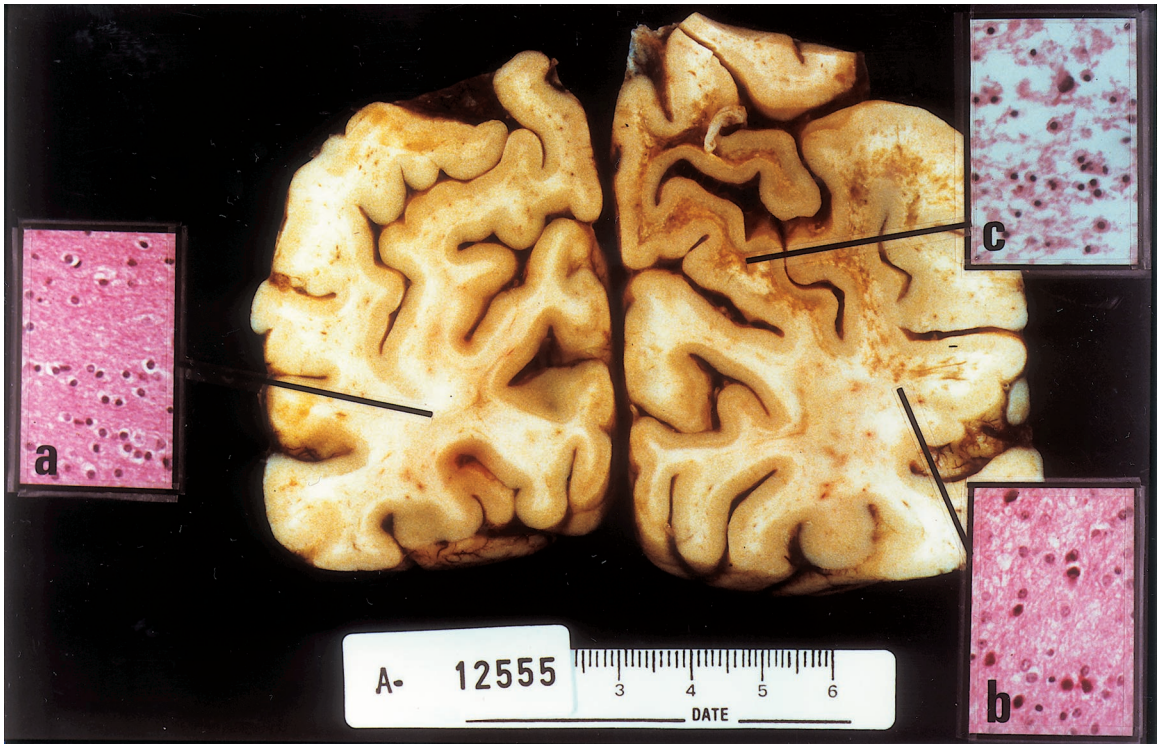

Figure 1 Photograph of a macroscopic coronal section of an AIDS brain with PML, to demonstrate regions with (**a**) absent, (**b**) mild or (**c**) severe involvement of PML, as judged by routine light microscopy. The left parietal lobe shows a large region of macroscopically visible, necrotizing PML. By light microscopy, this region reveals severe involvement by PML (insert **c**, H&E stain), and the adjacent region shows mild involvement (insert **b**, H&E stain). Within the same brain there are regions with no light microscopic signs of JCV-infection (absent PML) (insert **a**, H&E stain).

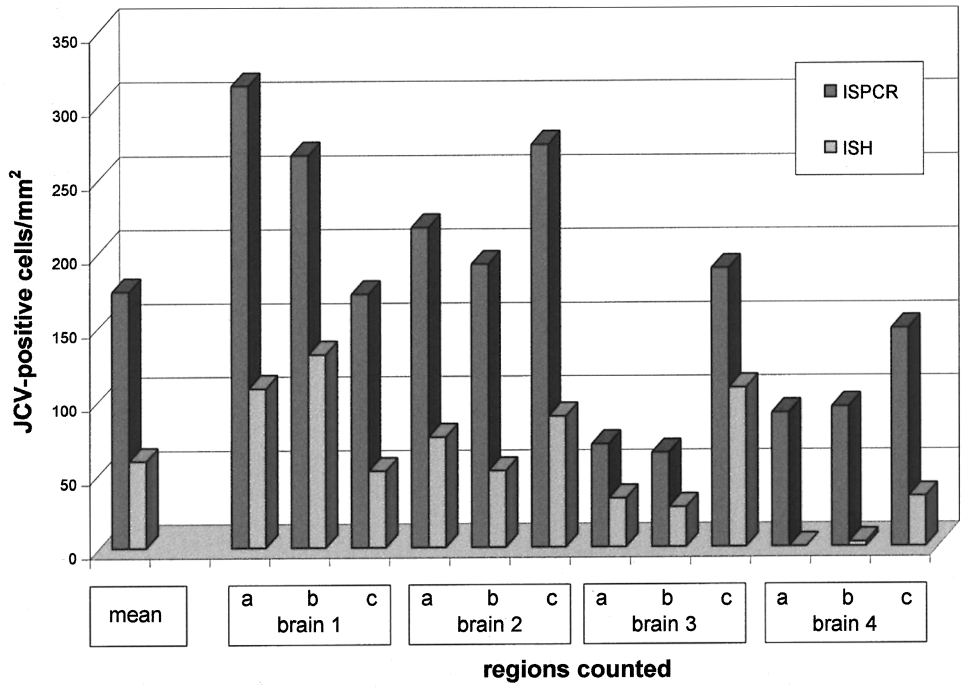

Figure 2 JCV-detection by *in situ* PCR versus ISH in parallel sections of moderately or severely infected brain tissue. The number of positive cells in three regions (a–c) of four different brains are shown. *In situ* PCR was consistently more sensitive than ISH; the number of positive cells after *in situ* PCR was 2–3-fold higher than after ISH alone. Note the remarkable difference in sensitivity between the two techniques in necrotic regions of severe JCV infection (brain no 4).

vicinity of blood vessels. Haematoxylin and eosin (H&E) staining allows approximate differentiation of only three degrees of JCV-infection: absent, mild, and severe (Figure 1). Based on the highly sensitive *in situ* PCR technique we propose a five-tiered staging of PML (Table 1).

(0) No evidence of JCV-infection by any method (uninfected region).
(i) Using *in situ* PCR, regions without evidence of JCV-infection by H&E or ISH demonstrated sporadic infected oligodendrocytes of normal size in white and sometimes also in grey matter.
(ii) In mildly infected regions without necrosis (Figure 3a,b) *in situ* PCR revealed some clusters of positive oligodendrocytes whereas ISH showed fewer, less dense clusters and parallel H&E-stained sections appeared normal.
(iii) In moderately infected regions (Figure 3a–d) an acute JCV-infection, using light microscopic criteria, could be identified most clearly with both DNA detection methods but was strikingly more prominent with *in situ* PCR than with ISH. In regions of central necrosis ample macrophages (recognizable by their round or oval and often eccentric nuclei with rounded and well defined cytoplasm), rare enlarged JCV-infected astrocytic nuclei and only a few JCV-positive peripheral oligodendrocytes were found. All cells were defined by their light microscopic morphology and not by immunohistochemistry. In a broad rim surrounding the necrosis collections of positively stained oligodendrocytes (Itoyama *et al*, 1982; Aksamit *et al*, 1986; Kuchelmeister *et al*, 1993; von Einsiedel *et al*, 1993; Dörries *et al*, 1998) were present in the neuropil, which still appeared intact. In the periphery of these foci the numbers of infected cells gradually decreased. In some regions we not only saw many more infected cells by *in situ* PCR than by ISH but also detected pronounced neuropil, i.e. myelin-associated staining, suggesting extracellular viral DNA, resulting from free virus released from damaged oligodendroglial cells at the end of the lytic cycle. This pattern supports the hypothesis of centrifugal spread of JCV-infection from the initial foci. On a single cell level (Figure 4a) most infected oligodendrocytes displayed enlarged JCV DNA-positive nuclei, others showed JCV DNA both in nuclei and homogenously stained cytoplasm. Occasionally, astrocytes revealed JCV sequences in their nuclei. In addition, some of these nuclei displayed a region of dense JCV DNA, probably representing either a prominently stained nucleolus or viral aggregates within the nucleus (Figure 4a). Surprisingly, on *in situ* PCR sections staining products forming a border suggestive of oligo-

Table 1 Proposed five histological degrees of PML

Degree of infection	*Observations*	In situ *PCR*	*ISH*	*H&E*
0 Uninfected	oligodendrocytes	normal unstained	normal unstained	normal
	myelin	intact	intact	intact
i Sporadic	oligodendrocytes	single infected cells normal size location: in white and rarely in grey matter	normal unstained	normal
	myelin	intact	intact	intact
ii Mild	oligodendrocytes	clusters of infected cells significantly more JCV-positive cells than by ISH	few JCV-positive cells	single oligodendrocytes with pathognomonically enlarged nuclei and altered staining characteristics
	myelin	pallor rarely vacuoles	pallor rarely vacuoles	pallor rarely vacuoles
iii Moderate	oligodendrocytes	centres: few oligodendrocytes, abundant lipid-laden macrophages margins: JCV-positive oligodendrocytes with staining product in nucleus or cytoplasm or both periphery: gradually decreasing numbers of JCV-positive cells	same pattern as for *in situ* PCR, but significantly fewer JCV-positive cells	abundant oligodendrocytes with pathognomonically enlarged nuclei and altered staining characteristics
	myelin	centres: frank necrosis, no remaining myelin margins and periphery: vacuoles and/or demyelination sometimes virus spill into neuropil	same pattern as for *in situ* PCR, but significantly less neuropil staining	same pattern as for *in situ* PCR, no virus spill
iv Severe	oligodendrocytes	only small number of JCV-infected oligodendrocytes disseminated lipid-laden macrophages	fewer JCV-infected oligodendrocytes than in *in situ* PCR or even none disseminated lipid-laden macrophages	rare oligodendrocytes disseminated lipid-laden macrophages
	myelin	confluent PML-lesions with extensive necrosis effacement of normal tissue architecture	same as for *in situ* PCR	same as for *in situ* PCR

dendrocytic cell silhouettes were discovered (Figure 4b). These cell contours appeared either as ghost cells or with an uninfected nucleus, and were located between necrotic centres and the surrounding rim of acutely infected oligodendrocytes. In contrast to JCV-infection patterns with both nucleic and homogenous cytoplasmic staining (Figure 4a), the oligodendrocytic cell silhouettes consist of a ring-like staining product implicating membrane-associated JCV DNA. These staining rings sometimes appeared fragmented, possibly representing cells in lysis.

(iv) In heavily infected regions with confluent JCV-foci and abundant macrophages, tissue architecture was no longer recognizable, and the brain tissue was fragile (Figure 5a, b).

Table 2 No significant difference in sensitivity after hot start and semi-hot start *in situ* PCR

Tissue		*Semi-hot start*	*Hot start*	*Ratio (Semi-hot start/Hot start)*
Brain	*Region*			
1	a	60[a]	55	1.09
1	b	48	40	1.20
1	c	83	88	0.94
2	a	135	140	0.96
2	b	109	99	1.10
2	c	27	34	0.79
Mean $\pm$ s.d.		77 ± 40	76 ± 41	1.01 ± 0.14

[a]Number of positive cells per mm^2.

Here, unexpectedly, *in situ* PCR demonstrated lightly stained oligodendrocytes in-

dicative of low JCV DNA copy numbers. The parallel ISH sections revealed either no positively stained cells or only very few, even paler cells.

Technical aspects of in situ *PCR on postmortem brain tissue*

Storage time of tissue before (up to 15 years) and after cutting (up to 2 years) did not affect the outcome of *in situ* PCR. For protease digestion, variation of the incubation time from 0 to 90 min at a constant protease concentration did not lead to diffusion of viral genome into the cytoplasm (due to overdigestion) and never to overall negative results (due to inefficient permeabilization). However, the highest number of marked cells was obtained with digestion times between 20 and 30 min. The duration of the wash step after endogenous peroxidase inactivation proved to be crucial. With 1×5 min, *in situ* PCR was less sensitive than ISH alone, whereas 4×10 min proved sufficient to reveal the superior sensitivity of *in situ* PCR. Classical hot start (both master mix and tissue preheated) and so-called semi-hot start (master

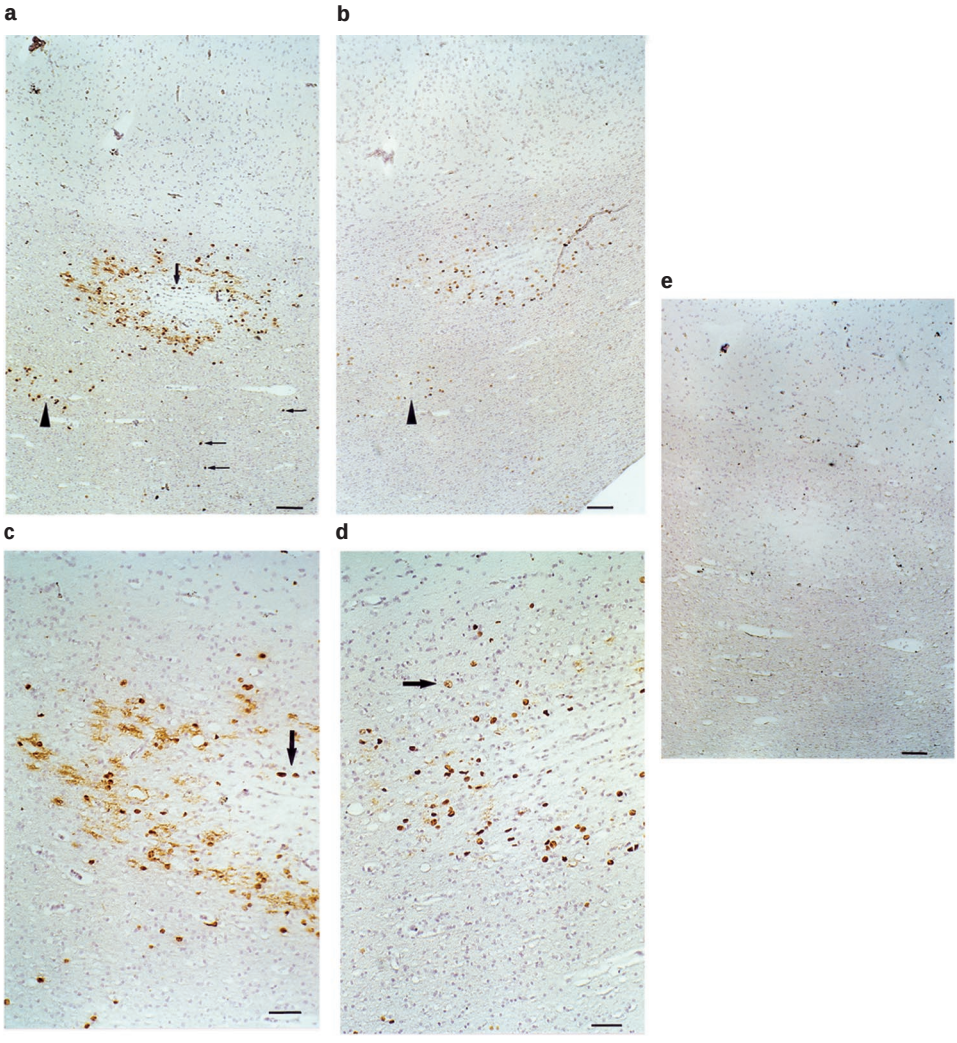

Figure 3 *In situ* PCR and ISH in brain tissue with PML. Necrotic region at the corticomedullary junction within white matter (grey matter at the top, white matter at the bottom), stage iii. Microphotographs of parallel sections after *in situ* PCR (**a**, **c**) and ISH (**b**, **d**), counterstained with haematoxylin. A collection of JCV-stained oligodendrocytes (brown DAB-staining product) in a broad rim surrounds the region of central necrosis. The number of infected cells gradually decreases in the periphery. Frank necrosis at centre of lesion with ample macrophages but hardly any positive oligodendrocytes. Note additional small infected foci without necrosis (arrowhead in **a** and **b**), and single infected oligodendrocytes (small arrows in **a**). The number of positive cells with *in situ* PCR exceeds that seen with ISH, staining intensity is greater with *in situ* PCR. Prominent signal is seen in the myelin. Two enlarged JCV-infected astrocytic nuclei (arrow in **a**, **c**) detected by *in situ* PCR. Enlarged infected astrocytic nuclei outside the necrotic centre (arrow in **d**). Scale bar=200 μm (**a**, **b**) and 100 μm (**c**, **d**). (**e**) Negative control on parallel section to *in situ* PCR and ISH (counterstained with haematoxylin), omission of probe. Scale bar=200 μm.

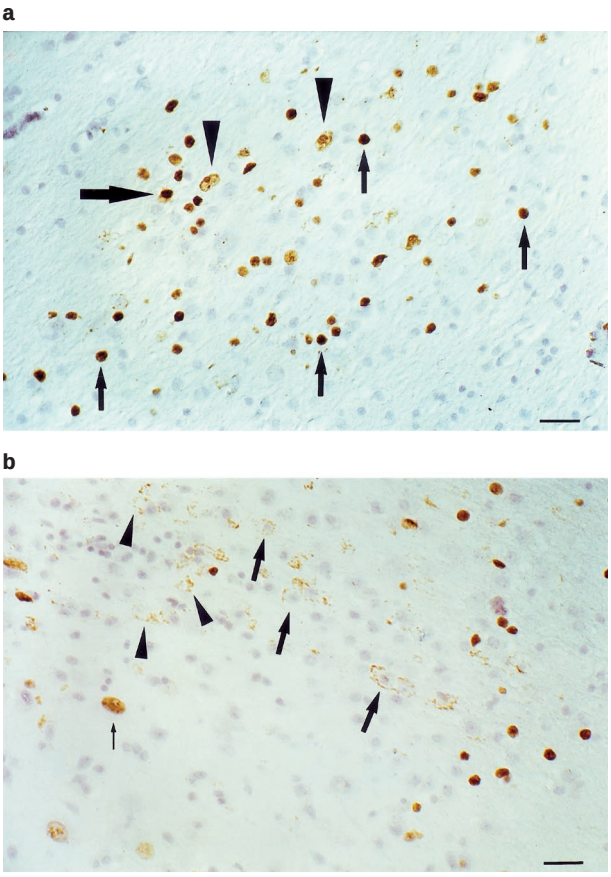

Figure 4 Single cell JCV infection patterns detected by *in situ* PCR (haematoxylin counterstaining). (**a**) Oligodendrocytes with enlarged infected nuclei (arrows), one oligodendrocyte with both infected nucleus and cytoplasm (large arrow). Two enlarged astrocytic nuclei with either prominently stained nucleoli or viral aggregates in the nuclei (arrowheads). (**b**) *In situ* PCR stain forming a border reminiscent of oligodendrocytic cell silhouettes, either with uninfected nuclei (arrows) or without nuclei (arrowheads). Infected astrocyte (small arrow) is located in the centre of necrosis. Scale bar=50 μm.

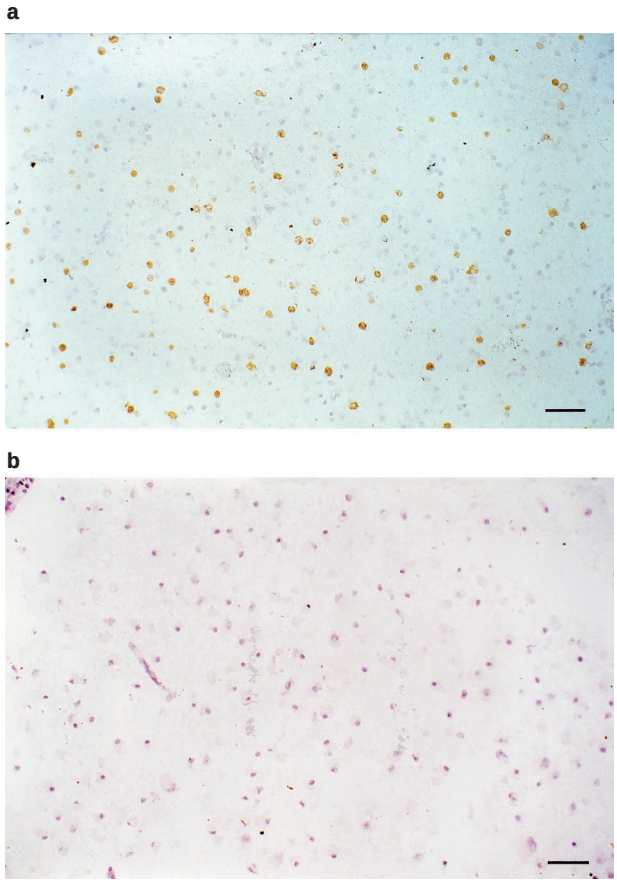

Figure 5 Superior sensitivity of *in situ* PCR over ISH in detecting JCV positive cells in a severely infected region (stage iv). Whereas abundant signal is seen by *in situ* PCR (**a**), none is seen in the parallel ISH section (**b**) (haematoxylin counterstain). Scale bar=100 μm.

mix at room temperature, preheated tissue) gave similar results (Table 2); the latter was preferred due to simpler, time-saving handling.

After 15 cycles evidence of amplification was detectable. Stronger signals were observed with 20 or 30 cycles. Since with 30 cycles tissue loss occurred and the risk of diffusion artefacts may increase (Komminoth *et al*, 1992), we routinely employed 20 cycles. An annealing temperature of 48°C was found to be optimal for *in situ* PCR in contrast to 50°C for solution phase JCV PCR. Addition of bovine serum albumin (BSA) to the master mix has been suggested to minimize background staining (Isaacson *et al*, 1994; Nuovo and Forde, 1995; Lewis, 1996). However, in our hands (0.1%, BSA fraction V, Bioproducts, Heidelberg, Germany) it caused non-specific staining.

A direct detection of *in situ* PCR products was tested by incorporation of digoxygenin (DIG)-labelled desoxyuracil triphosphate (dUTP) into the amplicons during the amplification reaction (Nuovo *et al*, 1992, 1993; Chen and Fuggle, 1993; Long *et al*, 1993; Komminoth *et al*, 1994b; Simon *et al*, 1997; Tolker-Nielsen *et al*, 1997; Tani *et al*, 1998; Yin *et al*, 1998). This method failed due to severe background staining in literally every cell and the entire neuropil, even when primers were omitted.

Discussion

The aim of this postmortem study of AIDS patients, whose death was directly attributable to PML, was a comparison of different JCV detection methods for the evaluation of intracerebral distribution of JCV infection. The intensity of cellular staining and number of JCV infected cells, as well as the intracellular distribution of viral DNA in brain tissue, was determined. For this purpose

tissue chosen from different regions with either absent, mild or severe involvement of PML by routine staining (Figure 1) was investigated with two techniques. It was not our aim to compare clinical symptoms with neuropathological findings, because for such examinations tissue derived from patients with recent as well as prolonged disease would be necessary. Grading of JCV-infection (degree 0–iv, Table 1) is based on histopathological observations.

With this investigation we have established *in situ* PCR on brain tissue from patients with PML in AIDS. JCV-sequences were detected with a significantly higher sensitivity than by conventional ISH. We found the *in situ* PCR method to be a reliable and reproducible technique when adequate controls are included. For our validation we limited the investigation to JCV, but in principle *in situ* PCR is applicable to various targets in all kinds of cells provided that the target DNA sequence is known.

The distribution of PML lesions was compatible with a haematogenous route of JCV dissemination to the brain (Chesters *et al*, 1983; Grinnel *et al*, 1983; Dörries, 1984; Myers *et al*, 1989; Azzi *et al*, 1996; Monaco *et al*, 1996; Pietzuch *et al*, 1996; Lafon *et al*, 1998). In accord with the findings of others (Chesters *et al*, 1983; Dörries *et al*, 1987; Telenti *et al*, 1990; Ferrante *et al*, 1995) there was no evidence of reactivated latent brain infection as suggested by some (Mori *et al*, 1991; Elsner and Dörries, 1992; White *et al*, 1992) since the small number of controls examined was consistently JCV-negative, controls included brain tissue of AIDS-patients without PML. These observations argue against the brain as a potential site of latency, and justify the examination of greater numbers of non-PML brain and extra-neuronal tissue from both healthy and immunosuppressed patients by *in situ* PCR, which will be the objective of future investigations.

The comparison of *in situ* PCR with ISH on parallel sections from regions with different degrees of infection (Figures 1–3) allowed us to propose a tentative scenario of virus spread in PML. Infections of the first degree (i) showed isolated JCV-positive oligodendrocytes that were sometimes present in intact neuropil and whose nuclei were of normal size and configuration without cytoplasmic stain. Some of these cells were detectable with ISH, as also described elsewhere (Aksamit *et al*, 1986, 1990; von Einsiedel *et al*, 1993) but many were only demonstrated by *in situ* PCR, due to less intranuclear JCV DNA. In advanced degrees (iii, iv) JCV leads to the typical picture of oligodendrocyte lysis and destruction of myelin (Figure 3a–d). During the course of PML areas of demyelination expand and necrotic areas coalesce (degree iv, Figure 5) (Aström *et al*, 1958; Richardson, 1961; ZuRhein and Chou, 1965; Brooks and Walker 1984; Aksamit *et al*, 1985, 1986, 1987; 1990; Anders *et al*, 1986; von Einsiedel *et al*, 1993), and certain intracellular infection patterns of oligodendrocytes and astrocytes are distinguishable. In infected oligodendrocytes JCV DNA was not only found in the nucleus but in some cells also within the cytoplasm. Ultrastructural studies have confirmed the presence of JCV-particles within these cell compartments (Mazlo and Tariska, 1980; Boldorini *et al*, 1993; von Einsiedel *et al*, 1993). Surprisingly, using *in situ* PCR, oligodendrocytes with cytoplasmic staining only were also seen (Figure 4b). The distribution of oligodendrocytes with different infection patterns illustrates at the light microscopic level that JC-virus infects the nucleus first. This stage is evidently followed by involvement of the cytoplasm before cell lysis finally occurs. This cytoplasmic infection pattern never occurred in early stages of sporadic or mild infection. We hypothesize, also in view of the often fragmented ring-like membrane staining, that cells with a border of brown staining product resembling the outline of oligodendrocytes, which were only discernible using *in situ* PCR, represent an end stage in the course of viral cell infection. It is also possible that cells with cytoplasmic staining are macrophages with phagocytized JCV-DNA. By their morphological appearance, however, these cells resemble oligodendrocytes rather than macrophages. Astrocytes are known to contain JCV DNA (Mazlo and Tariska, 1982; Aksamit *et al*, 1985, 1986, 1990; von Einsiedel *et al*, 1993); some of these cells revealed either prominent staining of the nucleoli or viral aggregates in the nucleus, not distinguishable by light microscopic criteria (Figure 4a).

Extensive staining was detected in myelin-associated neuropil to a significantly higher degree than observed by ISH. This virus 'spill' into the neuropil, possibly a correlate of papovavirus spread between myelin sheaths, as seen by electron microscopy (Kepes *et al*, 1975; von Einsidel *et al*, 1993), was limited to foci of acute infection (degree iii). Viral particles within myelin have been observed ultrastructurally in the same tissue (von Einsiedel *et al*, 1993). Whereas Weber and Major (1997) have postulated that virions remain cell-associated and spread by cell to cell contact, the finding of JCV DNA in the neuropil suggests that after cell lysis new cells can be infected by cell-free virus.

Due to its high sensitivity, *in situ* PCR in general bears a particular risk of nonspecific or false positive results. Analysis of formalin-fixed archival tissue poses the additional risk of nucleic acid degradation and loss of sensitivity. Therefore, some technical and methodological aspects of the procedure merit additional discussion. Time span between death and autopsy, duration of fixation (here 10–21 days) and embedding, storage time of tissue blocks (here 8–15 years) and sections (here up to 2 years) can all influence the integrity of DNA and thus its suitability for PCR (Komminoth *et al*,

1994a; O'Leary *et al*, 1994). Surprisingly, we had no problems amplifying the 207 bp JCV DNA fragment by *in situ* PCR. Both the short target sequence selected and the presence of multiple JCV copies in individual cells may have contributed to the high sensitivity despite the rather suboptimal tissue starting conditions.

Endogenous peroxidase was sufficiently inactivated by an incubation step in 1% hydrogen peroxide for only 30 min in contrast to most reports which recommend overnight treatment at concentrations ranging from 0.3 to 6% (Isaacson *et al*, 1994; Nuovo and Forde, 1995). We speculate that the short incubation time apparently sufficient for brain tissue is due to a low level of endogenous peroxidase. In the subsequent wash step, efficient removal of hydrogen peroxide as a potential inhibitor of Taq DNA polymerase was crucial for *in situ* PCR sensitivity. Numerous investigators mention the use of a hot-start for *in situ* PCR but the exact temperature or mode of application are not described (Nuovo *et al*, 1991b, 1992, 1993; Chin *et al,* 1992; Levin *et al*, 1996; Walker *et al*, 1996; Muciaccia *et al*, 1998). The time-saving semi-hot start used here resulted in the same sensitivity and specificity of *in situ* PCR as the classical hot start (Table 2). Our theoretical concern that the prolonged heating phase in semi-hot start would favour mispriming and primer-dimer formation did not hold true.

Although direct *in situ* PCR is faster and less laborious, indirect detection is the method of choice for us as well as others (Nuovo *et al*, 1991a, 1993; Bagasra *et al*, 1992, 1993, 1996, 1997; Chin *et al,* 1992; Komminoth *et al*, 1992, 1994b; Embretson *et al*, 1993; Long *et al*, 1993; Isaacson *et al*, 1994; Zehbe *et al*, 1994; Bashoff *et al.,* 1995; Walker *et al*, 1996; Naif and Cunningham 1996; Strayer *et al*, 1997; Xie *et al*, 1997). In our hands direct labelling of the PCR products always led to nonspecific results. The increased number of nicks and gaps in the DNA in postmortem tissue probably enhances nonspecific fill-in reactions by the DNA polymerase. It has been suggested that the problem of false-positive staining in direct *in situ* PCR can be overcome by several methods (Nuovo *et al*, 1993; Hamann *et al*, 1996; Peters *et al*, 1997; Simon *et al*, 1997), but their applicability is controversial (Komminoth *et al*, 1992, 19994b; Nuovo *et al*, 1992, 1993; Nuovo and Forde, 1995; Chen and Fuggle, 1993; Long *et al*, 1993; Cheng and Nuovo, 1994; Martinez *et al*, 1995; Ohshima *et al*, 1995; Hamann *et al*, 1996; Peters *et al*, 1997; Simon *et al*, 1997; Tolker-Nielsen *et al*, 1997).

For establishing *in situ* PCR for JCV DNA in brain tissue we paid special attention to the design and performance of effective and manifold controls, especially because the method is prone to yielding false results (Table 3). For initial validation 13 controls were found to be informative. In routine use eight controls were considered to be sufficient to assess consistency and reproducibility, rendering this highly sensitive and specific diagnostic method time and labour intensive.

Conclusions

In summary we have shown that *in situ* PCR allows the detection of JCV DNA with high sensitivity and specificity in formalin-fixed paraffin-embedded archival brain tissue. Although a rather detailed picture of JCV infection in brain tissue has already been obtained with conventional techniques such as routine H&E staining, ISH, immunohistochemistry and electron microscopy, *in situ* PCR adds

Table 3 Controls for indirect *in situ PCR*

	Controls	*Purpose*	*Results in comparison to* in situ *PCR samples*
1[a]	conventional ISH	successful amplification? successful hybridization reaction?	fewer positive cells, less intense staining
2[b]	healthy age-matched tissue	negative control	no staining product
3[b]	JCV-positive tissue	positive control	stained cells
4[b]	triplicate samples	consistent results?	similar staining patterns
5[b]	repitition of each run	reproducible results?	similar staining patterns
6[b]	template-DNA added to master mix	exclusion of cross-contamination	no additional positive cells, staining intensity unchanged
7[a]	solution-phase PCR on extracted DNA	false negative results? primers specific?	distinct band of 207 bp
8[a]	omission of antibody	endogenous peroxidase activity?	no staining product
9[a]	JCV DNA in master mix on blank slide and run in *in situ* PCR thermal cycler	amplification control	distinct band of 207 bp on agarose gel
10[a]	omission of DNA polymerase	successful amplication?	fewer positive cells
11[b]	air bubble control	direct comparison of areas with and without amplification	more efficient staining in areas with amplification
12[b]	omission of DNA probe	non-specific staining?	no staining reaction
13[b]	comparison of stained cells under and outside clip	amplification control	more positive cells under clip

[a]Included in each run. [b]For evaluation of the method and from time to time.

novel findings that may be relevant to disease pathogenesis. Our data led us to propose five degrees of PML infection in different regions of postmortem brain tissue.

It is tempting to speculate on the potential of *in situ* PCR to study presumed latent infection of other neurotropic DNA and also RNA viruses. However, it should be kept in mind that lower genome copy numbers and higher susceptibility of RNA for degradation may limit this approach and may require further technical improvements.

Materials and methods

Patients and processing of brain material
The brain tissue specimens for the study were obtained from nine AIDS patients with PML; the latter was the proximate cause of death in all patients. The clinical part of the study was done retrospectively, data was obtained from clinical charts. The clinical, radiographic, and pathologic features have been formerly described in detail (von Einsiedel *et al*, 1993), and are only briefly summarized here. Patients' ages ranged from 28 to 63 years with a mean of 43 years; only one was female. Risk factors were mostly homosexual contacts, followed by intravenous drug use; the one female acquired AIDS through a blood transfusion. Concurrent opportunistic infections of the CNS were present in some cases, PML dominated the clinical and pathological pictures (von Einsiedel *et al*, 1993). The brain tissue used as controls was obtained from a Caucasian, 29-year old, homosexual male AIDS-patient, and from three other age-matched patients with no underlying immune deficiency. Autopsies were performed at UCLA Medical Center, Los Angeles, USA, between August 1983 and July 1990. The brain tissue was fixed in 10% buffered formalin for 14–21 days prior to sectioning, the blocks were embedded in paraffin, and some of the tissues were cut onto slides up to 2 years before being studied. The blocks as well as the cut sections were stored at room temperature. Processing of brain material has been extensively described elsewhere (von Einsiedel *et al*, 1993). All nine brains investigated showed widespread evidence of PML. Regions with absent, mild or severe involvement of PML as judged by routine light microscopy and conventional criteria (Figure 1), (Aström *et al*, 1958; ZuRhein *et al*, 1965; Aksamit *et al*, 1985; von Einsiedel *et al*, 1993) were chosen for further study.

Sectioning procedure
The brain tissue blocks were cut into 6 μm thick sections and mounted directly onto wet silane-coated slides (Perkin Elmer Applied Biosystems, Foster City, USA). A separate microtome blade was used for each block. The tissue was heat-fixed on a hot plate at 55°C for 36–48 h.

Pretreatment of samples for in situ *PCR*
For deparaffinization the slides were immersed in xylenes twice for 15 min at room temperature, followed by rehydration of the tissue in decreasing concentrations of alcohol (100, 75, 50 and 25% ethanol for 5 min each) and then air dried. The cells were permeabilized in order to allow efficient reagent diffusion to the target nucleic acid. This was achieved by proteolytic digestion of the cell and nuclear membranes and cross-linked proteins using proteinase K. Monitoring of proteinase K digestion to control the optimal pore size of cell and nuclear membranes was achieved by the Situs™ control kit (Situs, Düsseldorf, Germany). Fluoresceine isothiocyanate- (FITC) labelled molecules designed to be of a size similar to that of the Taq DNA polymerase, which is the largest of the PCR reagents, were added to the slides together with the protease. Once the pores reach a size enabling the entry of FITC-labelled molecules into cells, the signal can be detected by fluorescence microscopy. 50 μl of proteinase K solution in reaction buffer (Situs™ control kit, Situs) at 1 mg/ml were applied to the slides and incubated at room temperature for 20–30 min. The reaction was stopped by rinsing the slides twice in phosphate buffered saline (PBS) at pH 7.4 for 5 min each, and the tissue was subsequently dehydrated through washes in graded ethanols (25, 50, 75 and 100%) for 5 min each. Endogenous peroxidase was inactivated by treating the tissue sections with 1% hydrogen peroxide in methanol for 30 min to prevent nonspecific staining. The slides were washed in PBS four times for 10 min each.

In situ *amplification*
To achieve a semi-hot start 50 μl of the PCR master mix at room temperature were applied to the preheated (70°C) slide on the assembly tool (Perkin Elmer Applied Biosystems). 50 μl of the master mix containing 1 μM of each JCV primer (5′-ACTGAGGAATGCATGCAGATCTAC-3′ nucleotides 4225–4249, 5′-TAGGTGCCAACCTATGGAACA-3′ nucleotides 4409–4429) (Arthur *et al*, 1989), 3 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.2 mM of each desoxyribonucleoside triphosphate (dNTP) and 10 units of AmpliTaq® DNA polymerase *IS* (Perkin Elmer Applied Biosystems) were added to each slide. The oval-shaped concave AmpliCover™ rubber discs, measuring 2.4 cm^2, and metal clips (Perkin Elmer Applied Biosystems) were assembled onto the slides, thus creating an air-tight reaction chamber to prevent evaporation during thermal cycling. After an initial denaturation step of 94°C for 3 min, DNA was amplified by 20 cycles of denaturation at 94°C for 2 min, annealing at 48°C for 2 min, and elongation at 72°C for 2 min. On completion of a final extension step at 72°C for 7 min, the AmpliCover™ discs and clips were dismantled and the slides rinsed in PBS.

In situ *hybridization*

ISH was performed for two different purposes, either following DNA amplification by *in situ* PCR as an indirect detection procedure, or in parallel alone as a conventional tool for JCV DNA detection in brain tissue sections. For *in situ* PCR the pretreated slides were subjected to thermal cycling or for ISH alone kept in PBS for the same time. All slides were rinsed twice in PBS, dehydrated through a series of ethanol washes (70, 90 and 100% ethanol) and pre-hybridized for 30 min at 37°C in a humidified chamber with 50 μl prehybridization solution containing 2 × saline sodium citrate (SSC), 50% formamide, 10% dextran sulfate, 10% Denhardt's solution, 0.5% Tween-20 and 250 μg/ml carrier DNA. After washing the slides in PBS, 50 μl of the hybridization solution composed of the prehybridization solution containing 1 μg/μl of a commercial DIG-labelled JCV Mad-1 probe (Kreatech Biotechnology, Amsterdam, The Netherlands) were added to the slides. The probe consists of a cocktail of oligonucleotides of 100–300 bp covering the 2.5 kb early region of the JCV genome. The probe and target DNA were denatured by incubating the slides at 95°C for 5 min on a hotplate, the heat quenched on ice and subsequently the hybridization reaction was allowed to proceed overnight at 37°C in a moist chamber. The post-hybridization washes consisted of a 15 min wash at 37°C with the differentiation reagent (Kreatech Biotechnology, The Netherlands) followed by three washes in PBS at room temperature for 1 min each.

DIG-labelled DNA was detected with a horseradish peroxidase-labelled anti-DIG antibody diluted 1 : 50 in 100 mM Tris-HCl (pH 7.5), 150 mM NaCl and 1% blocking reagent (Roche Molecular Biochemicals, Mannheim, Germany). Direct-affinity cytochemistry was carried out with diaminobenzidine (DAB) metal-enhanced hydrogen peroxidase substrate (Roche Molecular Biochemicals), yielding a precipitating brown reaction product at the site of DIG-labelled DNA. The results were monitored light microscopically and the reaction stopped by washing the slides twice in Tris-EDTA (TE) buffer. The tissue sections were subsequently counterstained with haematoxylin.

Controls for in situ *PCR*

To disclose false positive or false negative results up to 13 controls were included and processed simultaneously. The controls are summarized in Table 3. In particular, ISH alone is the most important parameter for demonstration of the superiority of *in situ* PCR over ISH and confirms the success of the hybridization reaction as such. Formalin-fixed brain tissue from age-matched patients without neurologic disease served as negative controls. Tissue sections formerly diagnosed as being JCV-positive by ISH and H&E staining (von Einsiedel *et al*, 1993) were included as positive controls, especially when the sample tissue was expected to be JCV-negative. A triplicate of samples was included in each run to verify the consistency of the results. Every run was repeated once to confirm reproducibility of the results. To evaluate the risk of cross-contamination and false positive results due to diffusion artefacts, we added 18.2 pg/μl template-DNA (plasmid containing the complete genome of JCV strain GS cloned over its unique *Eco*RI site, obtained from K Dörries, Würzburg, Germany) to the master mix and carried out *in situ* PCR on moderately JCV-infected brain tissue. Solution phase PCR using the same primers as for *in situ* PCR was performed on extracted DNA from parallel sections of *in situ* PCR specimens for the detection of false negative results. Omission of the enzyme-labelled antibody in immunohistochemical staining allowed the detection of endogenous enzyme activity. Solution phase PCR with 500 fg plasmid JCV DNA was performed on a blank slide to confirm that DNA amplification under *in situ* PCR conditions occurred. The omission of DNA polymerase functioned as an amplification control. A direct comparison of areas with and without PCR on the same slide was achieved by trapping a single air bubble covering about half of the tissue under the coverslip and thus preventing the master mix from reaching the tissue in this area. The omission of the DNA probe gave information on any nonspecific interaction of the detection reagents. Comparison of the staining pattern inside and outside the clip served as an inherent amplification control.

Solution phase PCR

For solution phase PCR tissue sections were digested with proteinase K (5 mg/ml) in the presence of lysis-buffer (100 mM TRIS, pH 7.6, 100 mM NaCl, 20 mM EDTA, and 5% SDS) for 1 h at 37°C. DNA was isolated by phenol chloroform extraction followed by ethanol precipitation, the pellet was dissolved in 50 μl sterile water, and 5 μl were used for PCR analysis. PCR was performed in a final volume of 50 μl containing 50 pmol of each JCV primer, 1.25 U AmpliTaq Gold DNA polymerase (Perkin Elmer Applied Biosystems, Foster City, California, USA), 3.5 mM $MgCl_2$, 400 μM of each desoxyribonucleoside triphosphate (dNTP) (Takara Shuzo Co. Ltd., Kyoto, Japan) and 1 × PCR-buffer (Perkin-Elmer Applied Biosystems). DNA was amplified in a thermal cycler (PTC-150 MJ Research, Watertown, Massachusetts, USA) by an initial activation step of 9 min at 95°C followed by 40 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C. Manifold precautions were undertaken to prevent PCR contamination, e.g. filtered pipette tips, separate rooms for DNA isolation and PCR, negative and positive controls in each run, use of AmpliTaq Gold to prevent re-opening of the tube for hot start PCR.

Evaluation of the slides
The cells were counted independently by two observers using a light microscope (Nikon Optiphot-2, Nikon GmbH, Düsseldorf, Germany). Cell type was assigned by light microscopic features. JCV-positive cells showed dark to light brown staining signals either in the nucleus, cytoplasm or both. Three different regions were evaluated on each tissue section. To compare the numbers of positive cells stained with different techniques on parallel slides, easily recognizable landmarks were used for orientation.

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