Papovaviruses in Humans

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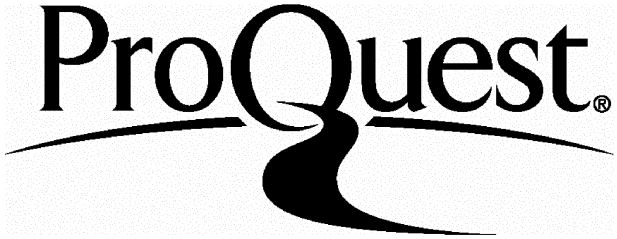

Abstract

Papovaviruses are a family of small, non-enveloped viruses of similar morphology that contain circular double-stranded DNA. They are divided into the two genera polyomaviruses and papillomaviruses on the basis of genome size and biological properties.

Human polyomaviruses comprise two agents, JC virus (JCV) and BK virus (BKV). After initial infection both persist indefinitely but are usually only associated with disease in immunosuppressed patients. JCV and BKV, together with simian polyomavirus simian virus 40 (SV40), have been associated with central nervous system disease. In particular JCV is associated with the demyelinating disease progressive multifocal leucoencephalopathy (PML), once considered a rare complication of immunosuppression in transplant recipients. With the advent of the AIDS pandemic, PML became of increasing importance in HIV-infected individuals. Human papillomaviruses (HPV) comprise a large group of inter-related but different viral genotypes. Some of these are termed low risk, causing warts at various sites in the body. Others, termed high risk, are aetiologically linked with squamous cell carcinoma, principally being implicated in the development of cervical carcinoma.

This thesis describes molecular methods developed for investigation of human papovaviruses. The initial part describes the development of molecular techniques for the detection and analysis of neurotropic polyomavirus sequences in samples from immunosuppressed patients. These methods were used to examine the correlation between polyomaviruses and clinical disease and to evaluate the use of molecular techniques in the diagnosis of PML. Nested PCR assays for JCV, BKV and SV40 were developed to determine the prevalence of polyoma sequences in CSF and brain samples. JCV was the only polyomavirus to have a direct correlation with PML. Detection of JCV DNA in the CSF of patients with a clinical diagnosis of PML was found to be 100% specific and 83% sensitive. Studies also looked at polyomaviruses present at other sites of the body and a comparison made of their genome sequences. The use of molecular techniques were shown to be of great value in diagnosis of PML

and the possible monitoring of the treatment of PML. However the introduction of highly active antiretroviral therapy (HAART) in the treatment of HIV-infected individuals has resulted in a dramatic reduction in the number of cases of PML in the UK and as a consequence, the intended study of clinical intervention was aborted.

The later parts of this thesis describes the development and application of similar molecular techniques for the detection and typing of HPV. PCR amplification was used to determine the prevalence of HPV at the cervix and amplicons were typed using a reverse hybridisation line probe assay. HPV DNA was detected in 80% of cervical smears taken from patients, often with multiple infection, attending a routine colposcopy clinic. Twenty one genotypes were identified in these samples confirming a need for methods to distinguish not only between high risk and low risk types but also between different genotypes infecting patients in this clinic.

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Publications

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Chapter 1: Introduction

1.1. Papovaviruses

Papovaviruses are a family of small, non-enveloped viruses of similar morphology. The term papovavirus was derived from the first two letters of three members of this group: rabbit *pa*pillomavirus, mouse *po*lyomavirus and simian *va*cuolating virus (now called SV40). The family is divided into the two genera polyomaviruses and papillomaviruses on the basis of genome size and biological properties. Both genera contain superhelical, double-stranded circular DNA within an icosahedral capsid. Polyomaviruses are the smaller genera, being 45nm in diameter, with approximately 5200 base pairs in the genome. The larger papillomaviruses are 55 nm in diameter with approximately 8000 base pairs in the genome. The fact that both genera are similar in appearance when viewed by the electron microscope led them to be included in the same family. However, these two genera are not related genetically and display different genetic organisation.

1.2. Polyomaviruses

1.2.1. Epidemiology of polyomaviruses

Currently there are 12 recognised polyomaviruses infecting humans, monkeys, hamsters, rats, cattle, rabbits, mice and parakeets Of these there are five which have been reported to infect primates (Table 1.1). Two human polyomaviruses have been described, JC virus (JCV) which was originally isolated from the brain of a patient with Hodgkins disease (Padgett *et al* 1971) and BK virus (BKV) which was originally

isolated from the urine of an immunosuppressed renal transplant recipient (Gardner *et al* 1971). Both of these viruses were named after the initials of the patient. In monkeys three polyomaviruses have been isolated, but only simian virus 40 (SV40) is considered relevant to humans because of its association with the early polio vaccines. Although the polyomaviruses are thought to display a narrow host range, *in vivo* sequence data have indicated that JCV and BKV are closely related to SV40 and lymphotropic papovavirus (LPV). Some studies have demonstrated the presence of SV40-like sequences in human tissue (Weiner *et al* 1972; Bergsagel *et al* 1992) and serological studies have indicated that humans have antibodies which cross-react with LPV proteins (Takemoto *et al* 1982). Thus, possible infection of humans with a primate polyomaviruses or the presence of a yet unidentified human polyomavirus has not been dismissed.

By adulthood the majority of individuals have been infected with JCV and BKV. Transmission is believed to be via the respiratory route and antibodies are detected in young children with the percentage of infection increasing with age. Data based on serological surveillance has indicated that at least 75% of adults have evidence of infection with JCV and the percentage can be higher with BKV. The majority of individuals infected do not have clinical symptoms and both viruses can persist indefinitely in the host. These human polyomaviruses are believed to remain in the kidney but have also been detected in the spleen, bone marrow and lymphocytes. Reactivation, leading to the excretion of JCV and BKV in urine, has been reported in many different groups including kidney and bone marrow transplantation recipients, pregnant women and those infected with HIV (human immunodeficiency virus). Although both BKV and JCV are shed in the urine it is only BKV which is associated

with diseases such as haemorrhagic cystitis and ureteral stenosis, while JCV reactivation is more commonly associated with central nervous system disease.

1.2.2. Genome structure of human polyomaviruses

The genome of the human polyomaviruses JCV and BKV are similar. They code for two non-structural proteins, large T and small t, three structural proteins, VP1, VP2 and VP3, and an agnoprotien. In SV40 the large T is involved in viral replication and it demonstrates transforming and transregulating activities (Keller and Alwine 1984). The JCV and BKV large T proteins are believed to exhibit the same properties. Less is known about the function of small t in human polyomaviruses, but in SV40 they have been associated with oncogenicity (Sleigh *et al* 1978). VP1-3, the capsid proteins, of which VP1 is the major one. The agnoprotien, named originally because its function was unknown, has been shown in SV40 to improve the efficiency of virus spread from cell to cell (Resnick and Shink 1986). The sequence around the origin of replication is termed the regulatory region. This represents the nucleotide positions 5014-276 in the JCV genome and within this region are found the promoter and enhancer elements for transcription. JCV and BKV both lack the ability to produce a DNA polymerase and therefore use the host polymerase for replication.

1.2.3. Human polyomaviruses and disease

Polyomavirus infection usually occurs at an early age, is transported to the target organ and can remain in that organ for the rest of life. In certain individuals under certain conditions both human polyomaviruses can be associated with disease. Disease occurs at primary infection or reactivation usually related to immunological impairments. Infection with BKV is believed to be involved in respiratory disease in

young children (Goudsmit *et al* 1982). This mild infection is believed to be due to primary infection because they showed seroconversion to BKV. Primary infection with BKV has also been associated with cystitis in children (Padgett and Walker 1983). BKV is often associated with haemorrhagic cystitis in bone marrow transplant (BMT) recipients and in a study by Arthur and colleagues (1986) it was shown that 16 out of 18 cases excreted BK virus in their urine. BMT patients undergo severe immunosuppresion before transplantation and are susceptible to reactivated polyomaviral infections. There are many other conditions where the patient is susceptible to polyomaviral reactivation due to immunosuppression such as 1) other types of transplants 2) chemotherapy in the treatment of malignancy 3) immunodeficiency diseases 4) infection with HIV 5) old age. In all these conditions the individual is susceptible to disease associated with BKV or JCV reactivation. The most serious and fatal disease is when the polyomavirus reactivates or infects the central nervous system and leads to progressive multifocal leukoencephalopathy (PML).

1.3. Progressive multifocal leukoencephalopathy

By far the most important clinical manifestation of human polyomavirus infection is PML. This disease of the central nervous system is characterised by enlarged oligodendrocyte nuclei and multifocal demyelination. PML was first described in 1958 (Astrom *et al*) and is almost inevitably fatal, with death occurring usually within one year after onset of symptoms. A viral cause for PML always seemed likely and in 1965 papova-like virus particles were seen in PML tissue by electron microscopy (Zu Rhein and Chou 1965; Silverman and Rubenstein 1965). Initially PML was thought to be to linked to both JCV and SV40 infections but it is now widely accepted that

the vast majority, if not all cases are solely due to JCV. Since its original isolation from a PML brain in 1971, JCV has since been isolated many times from CNS tissue and CSF of patients with PML, and there are reports of detectable virus in healthy brain (this will be discussed later). It is widely accepted that JCV does not cause PML in the absence of immunological impairment. Individuals, immunocompromised for reasons including chemotherapy prior to transplantation or because of disease (including AIDS) are susceptible to PML.

1.3.1. PML and AIDS

Originally PML was rare, affecting only occasional immunosuppressed or elderly individuals, but in recent years PML has become more common due to the increased number of HIV-infected immunosuppresssed individuals. With the outbreak of AIDS, the average age of PML-associated deaths has dropped dramatically (Holman *et al* 1991) and PML now accounts for approximately 4% of deaths in this patient group (Berger 1992). As a result of the higher incidence of PML in patients with AIDS there is speculation concerning possible interaction between HIV and JCV. An *in vitro* study has shown that the HIV-1 regulatory protein Tat can stimulate JCV replication in glial cells (Tada *et al* 1992).

1.3.2. Diagnosis of PML

An initial diagnosis of PML depends on neuroimaging such as Magnetic Resonance Imaging (MRI) and Computed Tomography (CT), combined with clinical data. Traditionally, a brain biopsy is used for the definitive diagnosis from which it is possible to identify the characteristic histological changes in the tissue and to detect the viral genome. Virus isolation for diagnosis is not practical because JCV is not

easy to grow in tissue culture and only replicates well in human foetal glial cells. Other methods for virus identification include detection of viral DNA by *in situ* hybridisation and detection of viral antigens by immunocytochemistry. Serology is usually unhelpful because in the majority of PML cases individuals were infected with the virus many years prior to onset of symptoms.

1.3.4. Re-activation of the JC virus

Whether PML is caused by reactivation of latent virus or by primary infection is still open to debate. Since infection of JCV usually occurs at an early age, PML is generally believed to be due to reactivation of the virus although there are a few cases of PML in immunosuppressed children which could be due to primary infection (Berger *et al* 1992; Newman and Frisque 1997). To help determine the mechanism of reactivation, genome sequence studies have looked for possible molecular alterations. Among JCV isolates from the brain of PML patients there is a considerable diversity in the regulatory region sequence, although the rest of the genome is remarkably conserved. It has been suggested that JCV undergoes genome alterations consisting of deletions and duplications when it is adapting to growth in brain tissue (Yogo *et al* 1993). When a rearranged genome arises it is presumed to have a replicative advantage over the archetypal type. It has been shown i*n vitro* that the rearranged JCV replicates more efficiently than the archetype in glial cell cultures (Daniel *et al* 1996).

Some studies have shown that only archetypal JCV is found in the kidneys and the rearranged type in the brain (Loeber and Dorries 1988), while others have shown the rearranged type in urine (Yogo *et al* 1991). A study by Ault and Stoner (1994) concluded that although the kidney contains mostly the archetypal form of JCV DNA,

there were a low percentage of rearranged sequences which were identical to those in the brain. It is generally believed that the archetype JCV is mostly confined to the kidneys and urine, while the rearranged type is detectable in a range of other tissues. Analysis of tissue from a paediatric PML patient (presumed primary infection) indicated that the archetypal sequence was in the brain (Newman and Frisque 1997). Hence this form of JCV can reach sites other than the kidney in immunosuppressed individuals but is likely to be present at low levels. In order to further our understanding of PML we need to determine the pressures which lead to the rearrangement of promoter/enhancer sequences and how this relates to persistence and activation of JCV in the kidney and CNS.

1.3.5. Latent JCV in the brain

There are conflicting reports concerning whether JCV persists in the brain of healthy individuals or is found only in the brain tissue of patients with PML. There are studies showing detectable JCV in normal brain tissue (White *et al* 1992; Quinlivan *et al* 1992; Mori *et al* 1992; Ferrante *et al* 1995). In contrast, others have concluded that JCV DNA can only be amplified from the brain tissue of patients with PML (Telenti *et al* 1990; Henson *et al* 1991; Mehal *et al* 1993). There are various explanations for these contradictory results. Firstly brain tissue extracts can inhibit PCR and therefore it is possible to fail to amplify the JCV DNA if it is present in low copy number. Second, and a generally more serious problem, is contamination of DNA from one sample to another prior to amplification leading to false positive results.

Whether reactivated or not, how does JCV transport itself to the brain? Not everybody agrees on this and it has been suggested that JCV could enter a healthy brain and reactivate in the CNS under conditions of immunosuppression (White *et al*

1992). Alternatively it could reactivate with immunosuppression and then transport itself to the brain. JCV DNA has been detected in peripheral blood lymphocytes of HIV-infected individuals (Tornatore *et al* 1992) and it has been hypothesised that JCV may gain entry to the CNS in B-lymphocytes of immunosuppressed individuals (Major *et al* 1992).

1.3.6. Treatment of PML

Progression of PML is rapid and, as it is a potentially fatal disease it is therefore important to consider an early treatment. The nucleoside analogue cytosine arabinoside, which is highly effective in preventing JCV replication in vitro (Tornatore *et al* 1994), has been most widely used though results have been mixed. Some reports have shown it to have no effect on clinical outcome (Castleman *et al* 1972), while others have shown that treatment with this drug has been followed by complete remission from PML (O'Riordan *et al* 1990). However, a placebo controlled study of cytosine arabinoside did not show any clinical benefit for the patients being treated with the anti-viral agent (Hall *et al* 1998). Other nucleoside analogues have been tried but most have had no clinical success, although there have been two reports showing a beneficial response to zidovudine (AZT) in HIV-infected patients with PML (Conway *et al* 1990; Singer *et al* 1994). The value of AZT in the treatment of PML without concomitant HIV is doubtful as polyomaviruses have no polymerase and use the host cell enzymes for replication. Should AZT prove to have a beneficial effect on progression of PML in HIV-infected patients this may be explained by suppression of HIV replication which, in turn, may be acting as a possible stimulus for JCV replication. A pilot study using alpha interferon was reported by Berger and colleagues (Berger *et al* 1992), but this gave no dramatic

effect on clinical outcome. A study using a combination of anti-retrovirals, including a protease inhibitor, has shown promising results. In this case study the patient had over two years remission from PML and regained 95% of his original function (Elliot *et al* 1997).

There is a search for other types of treatment for PML and one that holds some promise is camptothecin (a DNA topoisomerase inhibitor). Topoisomerases can change the conformation of covalently closed, circular DNA by introducing or removing supercoils. The JCV genome is a closed, circular supercoiled DNA molecule and *in vitro* camptothecin has been shown to prevent JCV replication (Kerr *et al* 1993).

1.3.7. BKV and Central Nervous System infection

BKV is usually associated with urinary tract disease, but has been detected in human brain tumours (Dorries *et al* 1987; De Mattei *et al* 1994). There was no clear evidence for a BKV association with the pathogenesis of these tumours and a study by Elsner and Dorries (1992) reported that BKV was present in 30% of normal brain tissue. In 1993, Vallbracht and colleagues detected BKV in brain tissue from an AIDS patient with meningoencephalitis and suggested that BKV infection is directly associated with severe neurological illness. BKV has been detected in the brain tissue of PML patients (Ferrante *et al* 1995), but it is not believed that there is any direct correlation with this disease. Other studies looking at polyomaviruses in the brain of PML patients failed to detect any BKV DNA (Schatzl *et al* 1994). Therefore if the BKV is latent in the brain it would be expected to be in low copy number and as yet there is no strong evidence for a role in CNS disease.

1.4. SV40 in humans

SV40 was first described in 1960 as a contaminant of batches of the then used polio vaccine (Sweet and Hilleman 1960). The virus was released from rhesus monkey cell cultures used for the production of poliovirus vaccines. In the period between 1955 and 1962 it was estimated that hundreds of millions of children and adults were given the contaminated vaccines. In 1962 this became a great concern when SV40 was found to transform human cells and induce tumours when injected into newborn hamsters (Shein and Enders 1962; Girardi *et al* 1962). In 1976, Shah and Nathanson carried out the first of many epidemiological studies to determine if this contaminated vaccine had any effect on humans by comparing groups of people who had been given the contaminated vaccine against control groups who had not. All these studies concluded that there was no increase in tumours within the group that had been inadvertently inoculated with SV40.

Two early cases of PML have been attributed to SV40 when Weiner isolated SV40-like virus from the brains of these patients (1972). However the role of SV40 in the pathogenesis of PML in man is still undefined since on re-examination of these cases, JCV DNA was detected in the brain tissue by *in situ* hybridisation. There have been other reports of SV40 associated with PML, but later studies show no evidence of detectable SV40 DNA in the brain of PML patients (Telenti *et al* 1990). In 1992 Bergsagel and colleagues (Bergsagel *et al* 1992) reported detection of SV40 DNA in half of 20 choroid plexus tumours and 10 of 11 ependymoma tumours. Following this study others began looking at other sites in the human body. In 1994 SV40 was associated with mesotheliomas (Carbone *et al* 1994), an asbestos related disease, but a later study ruled out any association (Strickler *et al* 1996). A study in 1996 to

determine the association of SV40 with human bone tumours found SV40 DNA in 41 of 200 osteosarcomas (Carbone *et al*). These findings have revived concerns about SV40 infection of humans and any association with malignancy. Therefore three questions need to be considered. Is the SV40 detected in these tumours connected with the use of contaminated polio vaccine? Although there is no evidence to suggest this to be the case, there is still a need to monitor the group as they increase in age. Second, is the SV40 detected in the tumours involved in the initiation of the tumour or is it just present in the tissue? Finally, are the SV40 results due to PCR contamination? The last point should be considered especially since SV40 is commonly used in research.

1.5. Papillomaviruses

1.5.1. Epidemiology of papillomaviruses

Papillomaviruses infect a large number of animals including primates, rabbits, mice, horses, cows, sheep, elk and deer. They are highly species specific and there are no examples of natural transmission to other species. Papillomaviruses infect and replicate in squamous epithelium and produce benign skin tumours in their hosts. Initially there was very little interest in them because they were not believed to be involved in serious disease, although this changed when they became associated with squamous cell carcinoma. Their association with cutaneous papillomatosis was established in 1933 by Shope and Hurst in the cottontail rabbit. In humans they have for a long time been associated with the common wart and are found at many different sites including the skin, genital tract, anal region, oral cavity and respiratory tract.

Classification of papillomaviruses has been attempted based on the degree of genetic relatedness, resulting in five supergroups A-E with some of these being further

divided in smaller groups. There are numerous human papillomaviruses (HPV) types, presently 84 are recognised and labelled by consecutive number. The number represents the order that they were discovered or established as a separate type. The types are divided on the difference in genome sequence and new types are only established after their sequence is determined.

Transmission of HPV occurs by some form of contact. Depending on the amount of infectious virus at the point of contact, it would be expected that intimate and frequent contact would be necessary to facilitate transmission. The point of contact may be through skin abrasions, sexual intercourse or at birth. Early work showed that genital warts were sexually transmitted (Barrett *et al* 1954). While in the late 1980's it was shown that HPV associated with malignant genital lesions were also transmitted sexually (Barrasso *et al* 1987; Campion *et al* 1988).

It was originally difficult to predict the prevalence of HPV because there were no serological assays to distinguish the different types. Also different HPV types tend to cause specific lesions. However, molecular techniques have been recently developed to detect HPV DNA, thus providing a better understanding of HPV prevalence and the role they have in disease. The prevalence of genital HPV infections is directly related to the number of lifetime sexual partners and age. The highest prevalence is found in sexually active women aged 15 to 25 years old (de Villiers *et al* 1987; Melkert *et al* 1993). In a study of college women, 46% had an HPV infection (Bauer *et al* 1991). The prevalence of genital HPV infections in males is believed to be similar but they have not been extensively studied.

1.5.2. Genome structure of human papillomaviruses

The genome organisation of the papillomaviruses differs significantly from that of polyomaviruses. The genome codes for six early (E) proteins; E1, E2, E4, E5, E6, E7 and two late (L) proteins; L1, L2. Although E4 is located in the early region, the product of this gene is expressed as a late protein and is involved in the release of papillomavirus particles. It has been suggested that E4 is involved in interacting with cytokeratins and therefore helps in virus release in the epithelium (Doorbar *et al* 1991). E1 and E2 are viral regulatory proteins involved in viral DNA replication. The E5, E6, and E7 proteins are involved in cellular transformation. In some HPV types the E6 proteins bind the tumour suppresser protein p53 (Werness *et al* 1990) which results in removing its control over the cell cycle. Also in these HPV types the E7 protein associates with the product of the retinoblastoma gene (pRB) (Dyson *et al* 1989), a tumour suppresser gene important in the negative control of cell growth. The E5 protein in some HPV types has been shown to interact with the epidermal growth factor (EGF) leading to an increase in the turnover and phosphorylation of the EGF receptors (Straight *et al* 1993). The late genes L1 and L2 encode the capsid proteins. These structural proteins are highly conserved among the papillomaviruses.

1.5.3. Human papillomaviruses and disease

HPV infect and replicate in squamous epithelium on both keratinised and mucosal surfaces. Clinical manifestations in humans range from benign papillomatous lesions to cancer. Most people are infected with the common HPVs, usually on the hands and feet and these can lead to warts. In general, warts are benign, self limiting and do not usually lead to any serious disease. HPV can cause skin cancer in people with the rare autosomal recessive disease epidermodysplasia verruciformis (Jablonska *et al*

1972). The genitourinary tract is the main reservoir of the mucosal HPVs, but they can also infect other mucosal surfaces such as the oral cavity. In the oral cavity HPV can cause warts, and in rare occasions these develop in the larynx which need to be removed by surgery. There are more than 25 HPV types that infect the genital tract. Some of these such as HPV6 and HPV11 produce genital warts, while others such as HPV16 and HPV18 are strongly associated with cervical cancer. The same HPV types are also associated with anal cancer especially in HIV infected men (Critchlow *et al* 1995). This study will focus on those HPV types that are suspected to be involved in cancer.

1.6. Cervical cancer

Cervical cancer is the second most common cause of cancer in women. World-wide there are about 450,000 cases of cervical cancer diagnosed annually (Perkin *et al* 1998). Although a number of risk factors have been associated with cervical cancer, HPV has been clearly established as the primary cause (Walboomers *et al* 1999).

1.6.1. High risk human papillomaviruses and cervical cancer

A large number of molecular and epidemiological studies conducted in the last 10 years have confirmed that infection with certain HPV types is the precursor event in cervical cancer (zur Hausen 1991; Schiffman *et al* 1993; Bosch *et al* 1993). Although in many cases infection with HPV is transient (Hildesheim *et al* 1994; Wheeler *et al* 1996) and the majority will not cause any problems. There are several known risk factors that influence being infected with HPV and the progression to cervical cancer. One of the important risk factors is the HPV genotype. Genital HPVs are defined as either high or low risk depending on their association with invasive cervical cancer.

By definition, infection with high risk HPV types increases the risk of cancer and a number of genotypes have been placed in this group (Table 1.2). Although there is still some debate as to how many HPV genotypes are classified as high risk types. Some of these high risk types are found more frequently in invasive cancers (e.g. HPV 16, 18, 31 and 45) and are considered high risk, while others found less frequently are sometimes termed intermediate risk. The genotypes in the low risk group are not believed to be associated with cancer but they have been detected in carcinomas (Bosch *et al* 1995). High risk HPV infection is not sufficient to induce an immediate carcinoma. Progression depends on persistence, immunologic factors and possible other co-factors. These co-factors include oral contraceptives and smoking.

1.6.2. Diagnosis of cervical cancer

Invasive cervical cancer is proceeded by a spectrum of abnormalities of the cervical epithelium. These abnormalities have been classified in various ways. Originally they were classified as cervical intraepithelial neoplasia (CIN) grades 1 to 3, or mild, moderate and severe dysplasia. The abnormalities are now classified as low and high grade squamous intraepithelial lesions (SIL). In order to prevent cervical cancer, screening programmes have been developed to detect these progressive abnormalities at an early stage. These involve taking a smear of cervical cells during a women's gynaecological examination in order to look for abnormalities. Any women who are picked up by the screening programme go for colposcopy for further diagnosis and will be monitored or treated depending on the progression of the cervical lesion. A biopsy is usually required before treatment. These screening programmes have been highly successful in reducing death from cervical cancer, but have not eliminated it. However there are problems with the methods used in screening, due to false

negatives because of errors in both the sampling and the interpretation of the smears. The screening programme is overburdened by borderline smears which are costly to follow up and cause anxiety to the women concerned (Raffle *et al* 1995). Also it must be remembered that these screening methods are not a diagnostic test and do not detect the cause of cervical cancer.

1.6.3. Detection of human papillomaviruses

Since the epidemiological data supports that HPV DNA can be recovered from over 95% of all cervical tumours (Schiffman *et al* 1993) it is necessary to determine the importance of DNA detection as a diagnostic tool. One of the problems with DNA testing is the high rate of positively in younger groups and this would mean that it could be expensive to use as a screening tool. Also, there are a large number of HPV genotypes and therefore a decision has to be made as to what HPV types to test for. There are various techniques used to detect HPV DNA including southern blotting (Low *et al* 1990), hybridisation (Clavel *et al* 1999) and the polymarase chain reaction (Morris *et al* 1990; Snijders *et al* 1990; Ward *et al* 1990; Kuypers *et al* 1993; Tieben *et al* 1993).

Southern blotting is very useful in HPV detection, but the high amount of input of DNA needed (which is not always available from a cervical smear) makes it unsuitable for screening. Hybridisation is a useful tool in diagnostic HPV detection and there is a commercial assay available called Hybrid Capture II (Digene). This assay involves denaturing the sample and then hybridisation with two target specific RNA probe mixtures. Each of these probes consist of a mixture of oligonucleotide sequences and are designed to detect either low or high risk HPV genotypes. The DNA/RNA hybrid is then detected with monoclonal antibodies to DNA/RNA. There

are many studies on the use of the polymerase chain reaction (PCR) for HPV DNA detection, but at the moment there are no commercially available assays. The primer sets designed for these PCRs will usually amplify DNA from either specific genotypes or from a broad spectrum of genotypes. The individual PCR assays for HPV genotype determination are highly specific (Cuzick *et al* 1994) although, because of the large number of HPV types, multiple PCRs would have to be performed which is time consuming. Therefore, it is an advantage to amplify a number of HPV genotypes with a multiplex PCR or by using universal primers. A number of universal primers have been described, but the MY09/11 (Resnick *et al* 1990) and the GP5/6 (Snijders *et al* 1990) primer sets have been most widely used.

The MY09/11 primer set uses degenerate bases to account for heterogeneity between various HPV types and amplifies a 450 base pair fragment in the conserved L1 region. The GP5/6 primer set uses two sequences, which in 1995 were modified by extending the sequence at their 3' ends to generate the primers GP5+ and GP6+ (de Roda Husman *et al* 1995). The resulting GP5+/6+ primer set amplifies a fragment of 150 base pairs in the L1 region. These primers are designed in a region of high homology and use a low anneal temperature to allow for mismatches. Recently a third primer system SPF10 has been developed by Kleter and colleagues (1998) which originally consisted of six sequences but was modified by the addition of four more sequences (Kleter *et al* 1999). These sequences are synthesised separately and mixed to form the primer sets. The SPF10 primer set amplifies a fragment of 65 base pairs in the L1 region.

Comparisons have been made between these primer systems. The sensitivities of MY09/11 and GP5+/6+ were comparable but there are differences in their ability to

amplify certain HPV types (Qu *et al* 1997). The SPF primers have been shown to be more sensitive than the GP5+/6+ primers (Kleter *et al* 1999).

These three primer sets described will all amplify a range of HPV genotypes but they do not distinguish between high and low risk HPV. Therefore various methods have been developed either to define the individual genotype or differentiate between high and low risk types. These include restriction fragment length polymorphism (Bernard *et al* 1994), detection by a cocktail of probes specific for high risk genotypes (Jacobs *et al* 1997) and methods depending on detection by specific probes immobilised onto strips (Gravitt *et al* 1998; Kleter *et al* 1999).

1.6.4. Treatment and prevention of cervical cancer

Persistent high risk HPV infections can lead to cellular deregulation which may then lead to high grade cervical lesions and ultimately cervical cancer. Diagnosis is carried out by colposcopy with cytology and requires a biopsy before treatment. Cervical cancer can be prevented by removing the lesion or ablation. Any treatment performed is dependent on the severity of the grade because low grade abnormalities do not always progress to invasive cancer. It was shown that only 14% of women with CIN 1 will progress to CIN3 (Syrjanen 1999) and therefore it is not always an advantage to treat early. Treatment is carried out using chemical, electrophysical or laser therapy.

Another strategy for the prevention of cervical cancer would be to immunise against HPVs. However this is not a simple task for many reasons. There is no suitable animal model for the growth of HPVs to test the efficiency of these vaccines. They would have to work on several HPV types to give sufficient protection against cervical cancer and it is not known if natural infection leads to a lasting immunity.

There has been some success in animal studies where both cattle and rabbits have been immunised against their own papillomavirus infections (Jarrett *et al* 1991; Lin *et al* 1992). In both these studies late proteins were used as antigens to prevent infection and therefore termed prophylactic. Other studies have been carried out on therapeutic vaccines (Cason *et al* 1993; Tindle *et al* 1994) which rely on destroying the HPV infected cells.

1.7. Diagnostic virology

Virus infections can be asymptomatic or cause serious disease. Most viral infections are acute and self limiting, while others can sometimes be fatal. Some infections are transient, while others are persistent for years and can lead to disease under certain conditions. Often disease due to a viral infection is picked up by the clinical symptoms. But in order to establish that the disease is due to infection, it is necessary to demonstrate virus, viral antigen or specific antibody. It is important that this diagnosis is made so that the patient can be treated and prevent the transmission of this infection. The actual preferred method of detection depends on the individual virus. Diagnosis of papovavirus infections in humans is difficult because they often have an asymptomatic course, serology is difficult for human infections and the virus often does not readily grow in conventional culture.

1.7.1. Detection by cultivation

It is possible to detect viral particles by electron microscope from cultivated BKV (Takemato *et al* 1979), although JCV is a difficult virus to grow in cell culture and only grows well in primary human foetal glial cells (Major *et al* 1984). HPV can not be cultivated in any conventional cell lines, although there have been reports of HPV

infection of xenografted foreskin tissue in nude mice (Bonnez *et al* 1992). HPV produced from this system has been shown to infect a neonatal foreskin epithelium *in vitro* (Smith *et al* 1995). It is possible to demonstrate papillomaviruses by the electron microscope from skin wart scrapings infected with HPV, although this may not be possible with mucosal HPV because of the limited number of particles present. Therefore the studies looking for diagnostic tools for JCV and mucosal HPV have mostly looked for viral genome or presence of a specific antibody response.

1.7.2. Detection by immunological methods

Serological techniques have played an important role in diagnostic virology. Species-specific polyomavirus antibodies can be distinguished from one another by neutralisation and haemagglutination inhibition (Pass and Shah 1982). Although serological studies on JCV and BKV have provided us with useful information on the epidemiology of these virus, it is often unhelpful in viral diagnosis because antibodies are present in the serum before the diagnosis of disease. Therefore it is necessary to look for significant titre changes or the IgM antibody levels that are linked with current infection. The presence of JCV specific IgM antibody has been reported in PML patients (Padgett and Walker 1983; Knight *et al* 1988) and renal transplant patients (Gardner *et al* 1984). Though the high prevalence of IgM in the sera of healthy persons limits its use in diagnosis (Knowles *et al* 1995). Of interest is the detection of intrathecal JCV antibody. One study showed that 67% of the CSF taken from patients with PML contained JCV antibody, while none was detected in CSF taken from any control patient (Knowles *et al* 1995).

There are very few serological tests for HPV because of the difficulties encounted in producing reagents for detecting specific antibodies against a single papillomavirus.

The problem is the difficulty in propagation *in vitro* and the high homology between the major capsid proteins L1 in the genotypes. However some success has come from recombinant viral-like particles (VLP), which have been produced using baculovirus vectors and insect cells (Kirnbayer *et al* 1992). Using these VLP, it has been possible to produce specific antiviral antibodies (Rose *et al* 1994). By tagging the antibody with a reagent that will visualise the specific antibody interactions it is possible to detect HPV in lesions.

The difficulties outlined above in the development or interpretation of immunological tests for papovaviruses have lead to studies focusing on using the new techniques for manipulating nucleic acids.

1.7.3. Detection by DNA-DNA hybridisation

DNA hybridisation has been widely used for the diagnosis of papovavirus infection. This method can detect a piece of viral DNA in a tissue by annealing a probe to it. The probe is a piece of DNA complementary or partially complementary to the viral DNA. To carry out the anneal step the target DNA has to be single stranded and therefore a denaturation step is carried out to separate the double stranded DNA. Denaturation can be carried out using chemicals or heating the DNA above its melting temperature. On removing the conditions causing denaturation, the single stranded DNA will anneal again and if any DNA in the tissue is homologous to the probe they will anneal to each other. The probe is labelled with a reagent that can be detected i.e. a radioactive molecule. This method has been used for diagnosis of PML. Using a probe radio-labelled (Dorries *et al* 1979) or biotinylated (Aksamit *et al* 1985; Houff *et al* 1989; Shapshake *et al* 1986) JCV has been detected from biopsy samples.

1.7.4. Nucleic acid amplification

The development of techniques for amplifying nucleic acids *in vitro* has revolutionised virology. There are many methods developed for sequence-specific nucleic acid amplification including the polymerase chain reaction (PCR), ligase chain reaction (LCR) and nucleic acid based amplification (NASBA). As mentioned earlier in this chapter there is a lot of interest in using PCR for the detection of both polyomaviruses and papillomaviruses. Therefore, since it is the main interest in this study, the next section will concentrate on PCR.

1.7.5. Detection by the polymerase chain reaction

The polymerase chain reaction (PCR) was first developed in 1985 (Saiki *et al* 1985). It allows the *in vitro* amplification of a particular DNA region. It basically involves the thermal separation of the two strands of DNA, the annealing of oligonucleotide sequences complementary to the ends of a defined sequence and enzymatically synthesising a new complementary DNA strand under appropriate conditions. This leads to the doubling of the original DNA copies, and if repeated, there is another two-fold increase. This can be repeated many times by using a thermal cycler leading to a huge amplification of DNA copies.

Two specific oligonucleotide sequences (primers) are synthesised using a DNA synthesiser. These primers are designed to be complementary to each end of the target DNA and depending on their purpose are usually 15 to 40 base pairs long, with a G+C content of 40 to 75%. DNA denatures at temperatures in excess of 90°C leaving single strand DNA. When it is allowed to cool to a specific annealing temperature the primers bind to the target sequence, one to the sense strand and the other to the antisense strand. It is then heated to a temperature (usually 72°C) suitable

for the incorporation of deoxynucleotide tri-phosphates (dNTPs) by a thermostable polymerase. This incorporation is extended along the single stranded DNA until a complete complementary strand of DNA is synthesised between the primers. This cycle of denaturation, anneal and extension is repeated until there is enough DNA to be detected. Using this two-fold amplification system it is possible to detect single copies of target DNA. The amplification is limited by the enzyme concentration which is being constantly used up by each reaction and eventually the exponential amplification rate changes to linear. This plateau effect is due to the concentration of the PCR products exceeding the polymerase concentration and the depletion of primer and dNTPs. Also, after too many cycles there is a danger of amplification artefacts. After the final cycle the temperature can be held at 72°C for a few minutes to help complete the extension. The final size of the PCR product (also called the amplicon) is determined by the distance between the binding sites of the two primers.

The most widely used method for PCR analysis is to estimate the fragment size by gel electrophoresis. The gel is stained with ethidium bromide and observed on a fluorescence transilluminator (Mullis *et al* 1986). Many other methods have been developed using labelled PCR primers and DNA probes which allow amplified products to be detected by colour, chemiluminescence, radiation or fluorescence.

It was the use of heat stable DNA polymerase in PCR (Saiki *et al* 1988) which opened this technique to many applications in molecular biology. This enzyme, originally isolated from the bacteria *Thermus aquaticus,* is stable at the high temperatures needed for denaturation and therefore does not need to be replenished after every cycle of the reaction. It soon became widely used in diagnostic virology including the detection of HIV (Coutlee *et al* 1991), hepatitis B virus (Sumazaki *et al* 1989),

hepatitis C virus (Brillanti *et al* 1991) and the detection of drug resistant viruses (Kaye *et al* 1992).

There have been many adaptations of this basic PCR principle. This study involves looking at some of these such as nested PCR, multiplex PCR and the use of consensus primers. Nested PCR will increase the sensitivity, as well confirming the specificity, of the target DNA. It involves designing primers which anneal internally on to the PCR product, so that a second round of PCR can be performed. Multiplex PCR can be used to amplify different DNA sequences on the same genome or DNA sequences from different organisms. The primers can be a mixture of totally different sequences or based on a consensus sequence. The consensus sequence can have total homology to all the target DNA or have a few mismatches in some of the individual targets. To overcome these mismatches the primers may be degenerate primers or a mixture of primers. The degenerate primers are synthesised by adding more than one base at each degeneracy resulting in a mixture of sequences, while the mixture of primers are synthesised separately and then mixed. In multiplex PCR the different targets can then be identified by size of the PCR product, the position of restriction enzyme sites or a collection of labelled probes.

1.8. Aims of the study

The principle aim of this study was to develop molecular methods for investigation of human papovaviruses and to look for correlation with disease. The first part was to develop methods for the detection of the human polyomaviruses, JC virus (JCV) and BK virus (BKV) in clinical samples from immunosuppressed patients. Both these viruses have been associated with central nervous system disease in humans and therefore studies were set up to assess their involvement. The main interest was to

look at patients with the demyelinating disease PML. Since the simian polyomavirus simian virus 40 (SV40) has been implicated in PML and there are concerns over the use of the SV40 contaminated polio vaccine, SV40 detection methods were also developed. PCR assays for JCV, BKV and SV40 were developed and used to determine the prevalence of neurotropic polyomavirus sequences in CSF and brain samples. The results were used to examine the correlation between polyomaviruses and clinical disease and therefore evaluate the use of molecular techniques in the diagnosis of PML.

It has been proposed that JCV undergoes genome alterations consisting of deletions and duplications when it is adapting to growth in brain tissue. Therefore one of the aims was to investigate if PML is associated with the rearranged type. To study this, samples were taken from other sites of the body and a comparison made of their genome sequences.

Screening programs for cervical cancer using cervical cytology have given significant reductions in both the incidence of and mortality from cervical cancer. However, there is still a good deal of ambiguity in the management of women with low grade abnormalities because of the poor sensitivity and specificity of cytology. Therefore the last aim of this study was to look at detection methods and characterisation for the human papillomavirus (HPV). Two consensus primer sets were used to amplify HPV by PCR and to determine the prevalence of HPV at the cervix. Since it is only certain high risk HPV that are associated with cervical cancer the amplified amplicons were genotyped using a reverse hybridisation line probe assay. The data from the two PCRs were compared and then the results correlated with cytology.

These studies were carried out over a number of years and therefore they are divided into five sections. Some of these studies are now archival and have been kept as they

were written at the time. A discussion has been added to each section and where possible a link has been kept between them. Therefore there has been no attempt to update some of these sections but this will be addressed in the general discussion. The reason for this being a partly archival thesis is that the criteria for the study changed. Originally it was to involve quantitative PCR for the JC virus and its use to monitor a clinical drug trial. However, the introduction of highly active antiretroviral therapy (HAART) in the treatment of HIV-infected individuals has resulted in a dramatic reduction in the number of cases of PML in the UK and as a consequence the intended study of clinical intervention was aborted.

Table 1.1. Polyomaviruses which infect primates

Virus	Natural host	Disease in natural host
JC virus	Human	Sub-clinical in immunocompetent individuals PML in immunosuppressed individuals
BK virus	Human	Sub-clinical in immunocompetent individuals Urinary tract infection in immunosuppressed individuals
Lymphotropic papovavirus	African green monkey	None identified
Simian virus 40	Rhesus monkey	PML-like disease in immunocompromised monkeys
Simian agent 12	Baboons	None identified

Table 1.2. Classification of genital Human Papillomaviruses by oncogenic risk

Oncogenic risk factor	HPV genotype
Low risk	6, 11, 34, 40, 43, 44, 53, 54, 55, 59, 61, 68, 70, 71, 74
High risk	16, 18, 31, 35, 33, 39, 45, 51, 52, 56, 58, 59, 66, 68

Chapter 2: Materials and general methods

2.1. Collection and treatment of samples

All samples taken from patients attending the London Hospitals were collected and prepared for storage on the same day. Samples collected in Miami were frozen there and then sent over on dry ice. Post mortem samples were collected on the day of the post mortem.

2.1.1. CSF

The volume of CSF collected varied from 200 - 1000µl. All but 100µl was put into 1.5ml tubes and spun at 15,000g for 5 minutes. The volume of CSF spun was recorded so that when the supernatant was removed the pellet could be resuspended in sterile water to make 5% of the original volume. The unspun CSF, supernatant and pellet were stored separately at -20°C.

2.1.2. Brain tissue

Brain tissue was taken at post mortem or by brain biopsy. The post mortem tissue was cut into 0.5cm^3 sections and placed in a cyrotube and snap freezed by immersion in liquid nitrogen. Fresh brain biopsy tissue was subjected to a DNA extraction immediately on arrival and then put through the required DNA detection tests within 24 hours. In a few of the smaller studies the brain tissue had been embedded in paraffin and stored at -20°C.

2.1.3. Blood

Two aliquots of blood were collected in Vacutainer™ (Becton-Dickinson) tubes. The 10ml of clotted blood was spun at 1300g for 10 minutes, the serum removed and stored at -20°C. The other aliquot of blood (15ml) was collected in a tube containing heparin in order to separate the mononuclear cells by density-gradient certification using Ficoll-Paque (Pharmcia Ltd. UK) by the following method. The blood was spun at 400g for 10 minutes, the plasma was removed and the cells resuspended in sterile phosphate buffered saline (PBS) to final volume of 15ml. At this stage 1ml blood/PBS was taken out and added to 1ml of glycigel which had been previously melted in a water bath at 37°C. This was stored at -70°C. The rest of the cell suspension was then layered onto 7.5 ml Ficoll-Paque in a sterile universal container and spun at 300g for 30 minutes. The mononuclear cells were removed from the PBS/Ficoll-Paque interface taking no more than 2ml and adding to 20ml of Rosswel Park Memorial Institute (RPMI) media (Gibco, Life Technologies, UK) in a universal container. This was spun at 300g for 10 minutes, the supernatant removed and the cells resuspended in a further 20ml of RPMI medium. This wash step was repeated. The pelleted lymphocytes were resuspended in 2ml of freezing medium, two equal aliquots placed into two cryotubes and stored at -70°C.

2.1.4. Urine

The volume of urine collected was approximately 2ml. One ml of the urine was spun at 15,000g for 5 minutes and the supernatant removed and stored with the cell pellet in separate containers at -20°C. Any unspun urine was also stored at -20°C.

2.2. DNA extraction and purification methods

2.2.1. Extraction of DNA by heat

The sample was heated in a boiling bath for 5 minutesand then placed on ice. This extraction was carried out immediately before the DNA amplification step.

2.2.2. Extraction of DNA from brain tissue

Each sample was minced using a sterile mortar and pestle which had been washed in 1M hydrochloric acid in order the remove any DNA from previous samples. The tissue was ground down to a fine powder and 1ml of sodium dodecyl sulphate (SDS) lysis buffer containing 100µg/ml Proteinase K was added directly to the pestle. This was mixed using a sterile Pasteur pipette and placed into a 1.5ml Eppendorf tube and heated at 60°C for two hours to inactivate any viruses. Then the sample was vortexed and heated at 37°C for 18 hours, with occasional mixing the sample by inversion. The lysis was stopped by boiling the tube for 10 minutes and placing on ice, if there were any large particles unlysed, they were removed by spinning at 3000g for 1 minute. The lysed cells were put directly into a DNA amplification stage or put through a phenol chloroform extraction.

2.2.3. Extraction of DNA from paraffin embedded brain tissue

One 5-10µm section was placed into a 1.5ml eppendorf tube and 400µl of xylene was added. The tube was vortexed vigorously for 2 minutes and centrifuged at 15,000g for 5 minutes. The xylene was removed and this step repeated twice. Four hundred microlitres of ethanol was added to the pellet, mixed gently to remove any residual xylene and centrifuged for 5 minutes at 15,000g. The ethanol was removed and this step was repeated before finally placing the tube without the cap into an incubator at

37°C for 10 minutes to allow any residual ethanol to evaporate. The pellet was resuspended in 600µl of SDS lysis buffer containing 100µg/ml Proteinase K, incubated at 60°C for 2 hours and then 37°C for 12 hours. A 100µl aliquot was removed, boiled for 10 minutes and put on ice. An ethanol precipitation was carried out on the remaining 500µl by adding 1ml of cold absolute ethanol and 150µl of 2M sodium chloride to give a final concentration of 0.2M.

2.2.4. Phenol chloroform extraction

The water saturated phenol (Rathburns, UK) was equilibrated to a pH of above 7.8 by the following method: Hydroxyquinilone was added to the phenol, to give a final concentration of 0.1%, the solution was then shaken and allowed to settle. The upper aqueous phase was removed and replaced with an equal volume of 1M Tris pH 8.3 and this step was repeated until a pH of 7.8 was obtained.

Two phenol chloroform extractions were performed using a reagent containing equal volumes of chloroform and phenol with 2% isoamyl alcohol to reduce foaming. Equal volumes of this reagent and sample were vortexed for 30 seconds and spun at 15,000g for 10 minutes. The aqueous top phase was removed for a further extraction with an equal volume of chloroform containing 2% isoamyl alcohol, vortexed for 30 seconds and spun at 15,000g for 10 minutes. The aqueous top phase was removed and the DNA precipitated by adding two volumes of cold absolute ethanol and 3M sodium acetate pH 5.2 to give a final concentration of 0.3M.

2.2.5. DNA extraction from samples stored in glycigel

The preserved sample was thawed and then spun at 14000g for 1 minute. An equal volume of lysis buffer was added mixed and spun at 14000g for 20 seconds. The

pellet was resuspended in 1 ml of lysis buffer and spun at 14000g for 20 seconds, this step was repeated until no unlysed cells remain. The pellet was resuspended in 100µl of extraction buffer containing 100µg/ml Proteinase K and incubated at 60°C for 2 hours. It was heated at 95°C for 10 minutes to denature the proteinase K and put directly into the PCR reaction mix.

2.2.6. Extraction of DNA by guanidinium

This was based on a method described by Boom and colleagues (1990). Nine millilitres of lysis buffer (L6), 50µl of silica particles, and 500µl of sample were added to a centrifuge tube and vortexed. After 10 minutes at room temperature the tube was vortexed again and centrifuged at 12,000g for 2 minutes. The supernatant was decanted and 1ml of wash buffer (L2) was added to the pellet. This was vortexed and transferred to a 1.5ml eppendorf tube and centrifuged at 12,000g for 2 minutes. The supernatant was decanted and was added to the pellet and centrifuged 12,000g for 2 minutes. This wash was repeated with 1ml 70% ethanol and then with 1ml acetone. The lid was removed and the vessels placed in a heat block at 56°C for 10 minutes. The sample was then eluted into 50µl of H_2O.

2.2.7. Extraction of DNA using Qiagen Spin Columns

The commercial QIAamp Blood Kit (Qiagen, Hilden, Germany) was used, Catalogue number 29104. This kit is designed for rapid purification of DNA from various body fluids. The procedure was carried out according to the manufacturer's handbook.

2.3. Synthesis of oligonucleotides

This section describes the preparation of oligonucleotide primers used for the polymerase chain reaction throughout this study. The design and optimisation of these primers will be discussed in the relevant section. The original primers were prepared in the laboratory using an oligonucleotide synthesiser, while the later ones were ordered from a commercial company (Oswel, Southampton, UK).

2.3.1. Preparation of oligonucleotide primers using an Applied Biosystems 381A DNA synthersiser

This synthersiser uses the phophoramidite method of oligonucleotide synthesis and was carried out according to the manufacturer's instructions. The oligonucleotides end up being attached to a controlled pore glass column and were extracted with fresh 30% ammonia solution at 4°C to minimise any evaporation. Two 5ml syringes were attached to each end of the column containing the newly synthesised primer. In one of the syringes 3ml of ammonia was placed and 0.5ml injected into the column and left for 15 minutes. This was repeated with successive 0.5ml portions until all the ammonia had passed through the column. The ammonia was then washed through the column 3 times from one syringe to another and collected into a glass vial. The protective groups were removed from the primer by incubation at 55°C overnight and the primer was stored at -20°C. The primers were purified from the ammonia solution by ethanol precipitation. One ml of ice cold ethanol and 150 μmoles of sodium acetate were added to 450 μmoles of primer in ammonia. This was mixed, and the tube placed at -20°C overnight. The DNA was pelleted by centrifugation for 10 minutes at 10,000g at 4°C. The supernatant was poured off and resuspended in 1ml

75% cold ethanol, spun for a further 2 minutes at 10,000g at 4°C. The supernatant was removed and the pellet resuspended in 100µl of H_2O.

2.3.2. Determination of primer concentration

The quantification of DNA was carried out by taking a reading (1 cm light path) with a spectrophotometer at a wavelength of 260nm and calculated using the equation 1 OD_{260}nm = 33 µg/ml single stranded DNA. Each primer was adjusted to 1 µg/µl with sterile distilled water and stored in aliquots at - 20°C. To confirm purity of the DNA a reading was also taken at 280 nm and the DNA presumed pure if the ratio OD_{260} / OD_{280} had a value of 1.8 (Sambrook *et al* 1989).

2.4. General PCR methods

This section describes the general PCR methods used in this study and PCR methods based on published data. The design and development of new PCR methods will be discussed in the relevant sections.

2.4.1. Standard PCR protocol

Each PCR was carried out in a total volume of 25, 50 or 100µl in a 500µl reaction tube. The extracted DNA was added in volumes of between 1µl to 25µl and overlaid with 100µl of light mineral oil. All initial PCRs were performed using a MJ research programmable heating block (Genetic Research Instrumentation, UK), while later ones were performed using a TC1 thermocycler (Perkin-Elmer Corporation, Connecticut, USA). Each PCR was preceded by a 4 or 12 minute 95°C denaturation step and followed by a final 5- 7 minute 72°C extension. PCR cycles ranged from 35

to 38 and when a second or third round was performed 1μl was transferred to a new reaction mix and put through 25 further cycles.

2.4.2. Contamination prevention methods

To avoid contamination of the PCR strict prevention measures based on those described by Kwok and Higuchi (1989) were applied throughout the study and by other colleagues using the shared laboratory areas. The PCR reaction mixes were set up in a laboratory which contained no amplified DNA and in the laboratory where PCR was carried out strict rules were applied to avoid PCR products returning to the set up area. On entering the post PCR laboratory disposable gloves and overshoes were put on and removed on exit. Nothing was ever removed from the post PCR laboratory except laboratory coats which were laundered separately from coats used in other laboratories. Separate sets of pipettes were used for the PCR reagents and samples.

2.4.3. Detection of human pyruvate dehydrogenase DNA by PCR

The pair of primers PDH-1 and PDH-2 (Table 2.1) was adapted from those designed by Koike (Rolfs *et al* 1992). This PCR had final concentrations of 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris (pH 8.3), 200 μM dNTP, 1.25 unit of AmpliTaq DNA polymerase (Perkin Elmer) and 100 ng of each primer. Initial denaturation was at 95°C for 4 minutes followed by 35 cycles of 95°C for 1 minute (denaturation), 56°C for 1 minute (annealing), and 72°C for 1 minute (extension), with an additional 7 minute extension. The products were electrophoresed through a 3% agarose gel, stained with ethidium bromide and visualised under ultraviolet light.

2.4.4. Detection of CMV DNA by PCR

The first round of this nested PCR used primers (CMV1 & CMV2) designed by Darlington and colleagues (1991) and the inner primers (CMV3 & CMV4) were developed by Wakefield and colleagues in 1992 (Table 2.1). The first round PCR was carried out in a 50μl volume containing 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris (pH 8.3), 200 μM dNTP, 1.25 unit of AmpliTaq DNA polymerase (Perkin Elmer) and 100 ng of each primer. Initial denaturation was at 94°C for 5 minutes followed by 35 cycles of 95°C for 1 minute (denaturation), 58°C for 1 minute (annealing), and 72°C for 1 minute (extension), with an additional 7 minute extension. After completion of the 35 cycles, 1µl of the first round product was transferred to a fresh PCR mixture containing the "inner" (nested) primers. The second round PCR reaction was carried out in a total volume of 25µl with the same conditions as in the first amplification, except the anneal temperature was lowered to 50°C, the primer concentration was doubled and the initial 4 minutes denaturation at 94°C was omitted. After 25 cycles, the products were electrophoresed through a 3% agarose gel, stained with ethidium bromide and visualised under ultraviolet light.

2.5. Sequencing

Manual dideoxy sequencing (Sanger *et al* 1997) was carried on brain and urine samples. Design and conditions of the PCRs to amplify DNA to obtain a template are described in chapter 6. The basic methods for purification and sequencing of single stranded biotinylated PCR products are described here.

2.5.1. Purification of biotinylated PCR products

The biotinylated PCR products were captured onto streptavidin coated magnetic beads [Dynabeads (Dynal, Skoyen, Norway)]. Seventy microlitres of the PCR product were added to 20μl of beads. Then 60μl of 2x Bind and Wash solution was added and incubated at room temperature with gentle agitation for 15 minutes. Any captured DNA was pelleted by a magnetic field created by a specially designed rack (Dynal, Skoyen, Norway). The pellet was washed with 40μl 1x Bind and Wash solution. The DNA duplex was separated by incubating for 10 minutes at room temperature with 8μl of NaOH. The eluted non biotinylated strand was removed, neutralised with 4μl of 0.2M HCL and 4μl of TE pH 7.4, and then stored at -20°C.

The biotinylated strand then was subjected to the following washes, first with 50μl of 0.1M NaOH, second with 40μl of 1x Bind and Wash solution and finally with 50μl of TE buffer. After removing the TE buffer the DNA was resuspended in 10μl of water.

2.5.2. Manual dideoxy sequencing

The sequencing reaction was performed as a modification of the Sequenase Version 2.0 T7 DNA Polymerase Sequencing Protocol (United States Biochemical, USA). Briefly, this was as follows.

In a 500μl centrifuge tube 100ng of sequencing primer, 500ng of single stranded DNA, 1μl DMSO, 2μl 5x sequenase reaction buffer (United States Biochemical, USA) were mixed and the volume made up to 10μl with water. The primer was then annealed to the template DNA by denaturing the samples at 70°C for 5 minutes and then cooling to 25°C over 30 minutes in a TC1 thermocycler.

While this anneal was taking place a labelling reaction mix was prepared containing per sample; 1μl DTT, 0.5ul ^{35}S dATP (1000 Ci/mM), 2μl of labelling dNTP mix

(diluted by 1/30) and 0.2μl sequenase enzyme (United States Biochemical, USA). Also a V bottomed microtitre plate was prepared, containing the termination reactions with four separate wells, labelled G A T C, for each sample. Each well contained the appropriate dideoxy termination mixture, 2.5μl of the ddNTPs and 0.2μl DMSO per well. The microtitre plate containing the termination reactions was covered with a plate sealer and placed on top of wet tissues on a hot block at 50°C.

The annealed sequencing primer and template were then pulse spun to bring down any condensation. Four and a half microlitres of the labelling reaction mix was added to the 10μl of the annealed sequencing primer and template and incubated at room temperature for 5 minutes. Three microlitres of the completed labelling reaction was added to each pre-heated termination reaction (wells labelled G A T C). After 2 minutes, 4 μl of stop solution was added to each well. This was performed with the samples heated at 50°C on the hot block. The plate was covered with a plate sealer and stored at -20°C until the samples were run on a sequencing gel.

2.5.3. Sequence electrophoresis

The reaction products were run on a wedged 8% polyacrylamide gel. The glass plates were carefully cleaned by washing with 70% ethanol and acetone before use. The reaction products while still in the microtitre plate were incubated on a hot block at 90°C for 5 minutes to denature the DNA. Three and half microlitres of sequenced products were loaded on to the gel and run at 70W for 2 hours in 1x TBE buffer. Sodium acetate was then added to the buffer in the cathode chamber to a final concentration of 1.0M and the gel run for a further one hour. Once finished the gel was removed and fixed on 3MM filter paper (Whatman, Maidstone, UK) with 10%

methanol and 8% acetic acid. The gel was incubated twice at room temperature for 15 minutes in fixing solution. The gel was then dried under vacuum for 2 hours using a gel drying apparatus. Finally the gel was placed in an autoradiograph case and exposed to X ray film at room temperature for 24 hours. It was then developed in a automatic developer.

2.6. Media and buffer recipes

PCR reagents

PCR reaction buffer (10 X RB)

5.0×10^{-1} M KCl

2.0×10^{-1} M Tris

1.5×10^{-2} M $MgCl_2.6H_2O$

pH 8.4

Autoclaved

Gel loading buffer

30% (v/v) glycerol

0.25% (w/v) xylene cyanol

TAE (50x)

1.0 M Tris

6% acetic acid

5.0×10^{-2} M EDTA

(from pH 8.0 stock)

Agarose gel

1 X TAE (from 50X stock)

2% (w/v) agarose

0.05% (w/v) ethidium bromide

Sequencing reagents

Bind and Wash solution

10 mM Tris-HCl pH 7.5

1 mM EDTA

2.0 M NaCl

Labelling dNTP mix

7.5 mM dCTP

7.5 mM 7-dGTP

15 mM dTTP

Termination mixes

160 mM dATP

160 mM dTTP

80 mM dCTP

40 mM dGTP

In addition to the above reagents each termination mixes contained the following concentrations of dideoxy nucleotides:

'A' termination mix	8 mM ddATP
'T' termination mix	8 mM ddTTP
'G' termination mix	4 mM ddGTP
'C' termination mix	4 μM ddCTP

Stop solution

98% Deionised water

10mM EDTA (pH 8.0)

0.025% Xylene cyanol FF

0.025% Bromophenol blue

TBE buffer

20 x 1M Tris base

1M Boric acid

2.0 mM EDTA NaH_2O

Polyacrylamide gel

7.5g urea

10ml TBE

20ml acrylamide mix (40%)

150mg $(NH_4)2SO_4$

15μl Temed

Make up to150mls in H_2O

General reagents

Complete RPMI

2.0 X 10^{-3} M L-glutamine

100 units / ml penicillin

100 µg / ml streptomycin

2.5 µg / ml fungazone

10% (v/v) FCS

made up in 1 X RPMI

(Dutch modification)

Freezing medium

10% (v/v) DMSO

30% (v/v) FCS

Made up in 1 X RPMI

(Dutch modification).

L2 Wash buffer

5M GuSCN

50mM Tris hydrochloride pH 6.4

L6 Lysis buffer

5M GuSCN

50mM Tris hydrochloride pH 6.4

20mM EDTA

1% Triton X-100

Glycigel freezing medium (for 1 litre)

375 ml glycerol

1.8g EDTA

15g gelatin

5.4g NaCl

1.0g sodium azide

make up to 1 litre with distilled water

heat to 70°C until all gelatin dissolved, cool and aliquot

Glycigel lysis buffer

1 X 10^{-2} M Tris-HCl

(pH 7.5)

1% (v/v) Triton X100

3.2 X 10^{-1} M sucrose

5.0 X 10^{-2} M $MgCl_2$

Sterilise Tris-HCl / sucrose / $MgCl_2$ and adjust pH before addition of Triton

Glycigel extraction buffer

1 X 10^{-2} M Tris-HCl

(pH 8.3)

5.0 X 10^{-2} M KCl

2.5 X 10^{-3} M $MgCl_2$

0.0001% (w/v) gelatin

0.45% (v/v) Tween 20

0.45% (v/v) Nonidet P40

Sterilise Tris / EDTA / NaCl by autoclaving and adjust pH before addition of gelatin / Tween-20 / Nonidet P40

SDS-extraction buffer

1.0 X 10^{-2} M Tris

2.5×10^{-2} M EDTA

1.0×10^{-1} M NaCl

0.5% (w/v) SDS

100 µg /ml proteinase K

pH 8.0

Sterilise Tris / EDTA / NaCl by autoclaving and adjust pH before addition of SDS

Add proteinase K just prior to use.

Phosphate buffered saline (PBS)

1.5×10^{-1} M NaCl

1.5×10^{-3} M KH_2PO_4

6.3×10^{-3} M $Na_2HPO_4.12H_2O$

2.6×10^{-3} M KCl

pH 7.2

TE buffer

1.0×10^{-2} M Tris

1.0×10^{-3} M EDTA

Table 2.1. PCR oligonucleotide primers for human pyruvate dehydrogenase and CMV

Oligonucleotide	Sequence (5'→3')	Nucleotide position
PDH-1	GGGTATGGATGAGGAGCTGGA	4296-4316
PDH-2	TCTTCCACAGCCCTCGACTAA	4482-4462
CMV1	GAGGACAACGAAATCCTGTTGGGCA	1942-1966
CMV2	GTCGACGGTGGAGATACTGCTGAGG	2091-2067
CMV3	ACCACCGCACTGAGGAATGTCCAG	1967-1989
CMV4	TCAATCATGCGTTTGAGGAGGTA	2066-2044

Chapter 3: Development of PCR methods for the detection of polyomaviruses

3.1. Introduction

In the development of PCRs for the detection of JCV, BKV and SV40 it was important to design the primers in an area of the genome that is highly conserved. Since these three polyomaviruses showed a high degree of homology in their genetic sequence, it is also important to pick an area which shows enough variation to be able to design primers which can be used to detect them individually. Reports have shown the large T region to be highly conserved in all three polyomaviruses (Frisque *et al* 1984) and therefore, initial analysis for primer design was undertaken in this area.

Because of the possibility of there being mixed populations in any one sample, the use of multiplexing was taken into account in the design of the PCRs. This is because considerable savings of time and effort can be achieved by simultaneously amplifying sequences in a single reaction. Two different approaches were made to amplify these polyomaviruses. The first approach was to design consensus primers which amplified all three polyomaviruses in a single round PCR and then use specific primers to amplify each type of polyomavirus in a second round semi-nested PCR. The second approach was to design three specific sets of primers which, after nested PCR amplified DNA of different size and therefore could be differentiated by electrophoresis on an ethidium bromide-stained agarose gel. This would allow the three sets of primers to be used in a multiplex PCR. This chapter describes the design of primers for these two approaches by using computer analysis and the use of these

methods to detect polyomaviruses in clinical samples. This section also describes studies looking at the specificity of the PCRs and the establishment of controls to standardise the sensitivity.

3.2. Computer analysis

The original work on primer design was achieved using a computer programme called PC/GENE (IntelliGenetics Inc. California, USA). This software contained the European Molecular Biology Laboratory (EMBL) data bank and was updated every month via a CD-ROM. The limitation at the time of the study was that this database contained few JCV and BKV nucleotide sequences. At the time the EMBL database did not include a whole JCV genome and therefore, one was exported from another database Microgenie (Beckman, USA). Because of limited sequence data available for JCV and BKV, it was decided to set up library files on PC/Gene which could then be used to screen newly designed primers.

Although all the initial primer design was achieved with PC/Gene, during the later stages of the study more advanced methods of computer analysis became available. In particular, the use of the internet gave better access to databases and the whole process could be achieved in a fraction of the time. Some of these methods have been included in this chapter when they were used to confirm or re-evaluate the original primer design.

3.2.1. Alignment of nucleotide sequences

The genome sequences of the JCV, BKV and SV40 were 5130, 5153 and 5243 base pairs long respectively. The whole of each genome was aligned to each other using NALIGN on PC/GENE which is based on a method developed by Myers and

Miller (1988). The genome similarity based on the number of identical bases was 72-76% between the three viruses (Fig 3.1). A region in the large T of approximately 700 bases long was chosen for further analysis. Using these alignments areas of high homology were found between the genetic sequences which were used for design of the consensus primers. The alignments showed areas that had sufficient mismatch between the sequences of the genomes to be able to differentiate between the three polyomaviruses for the design of virus-specific primers.

3.2.2. Restriction enzyme maps

Restriction endonucleases were used to validate the specificity of the amplified PCR product. The specificity of a PCR amplification product was determined by analysing the restriction fragments generated by digest with various restriction endonucleases. Using the programme RESTI (PC/GENE) an enzyme map was created for the 4000-5130 region of JCV, the 4000-5153 region of BKV and the 4000-5243 region of SV40. This programme displays the restriction enzyme sites by order along the sequence in alphabetical order. A search using all the enzymes on the database was made to find all possible cleavage sites within these regions.

3.2.3. Hairpin loops

To avoid areas of the genome where possible hairpin loops could be formed in nucleotide sequences a search using the programme HAIRPIN (PC/GENE) was carried out on the 4000-5130 region of JCV, the 4000-5153 region of BKV and the 4000-5243 region of SV40. In JCV it was found that sequences at 4838 and 4947 could give rise to hairpin loops and therefore care was taken to design primers which

avoided these areas. No areas were found in BKV or SV40 which could lead to possible hairpin loops.

3.2.4. Library files

Library files contained the names of individual sequences taken from databases. The advantage was that you could combine information from databases and speed up homology searches. From the alignment of the three polyomaviruses (section 3.2.1.), an oligonucleotide sequence of 20 bases (CP3) was chosen which had a high homology with all three sequences (Table 3.1). Using the programme QGSEARCH (PC/GENE), the EMBL database was searched for sequences matching CP3, allowing for 5 mismatches. This search picked up 5 viral sequences which included 2 BKV (PABKVDN, PABKVMM), 2 SV40 (PAPSV4CG, SV40XX) and one herpes simplex virus (HSV, NHE1CG_02). No JCV sequences were picked up by this search, presumably because it did not contain any at the time. Therefore the JCV sequence (PAPPLYCG1) found on the database Microgenie was combined with the 5 sequences from PC/GENE to create the library file JCBKSV. At approximately 600 base pairs downstream from CP3 a second olignucleotide sequence (CP4, Table 3.1) was chosen which also had a high homology with the three aligned polyomaviruses. The library file JCBKSV was searched for sequences matching CP4, allowing for 5 mismatches. Only the 5 polyomaviruses were picked up in this search.

3.2.5. Homology sequence searches

Oligonucleotide sequences were designed for use in the detection of polyomaviruses by PCR. These sequences were screened against various databases or library files. The initial searches were by QGSEARCH against the library file JCBKSV to find

sequences that were either complementary to all 3 polyomaviruses or specific to one of them. The chosen sequences were then screened against the EMBL database. Sequences designed later in the study were screened by FASTA (Pearson and Lipman, 1988) on the world wide web accessed from the European Bioinformatics Institute (EBI) home page. All primers designed to be used in the same PCR were checked for complementarity between their sequences by the programme NALIGN.

3.3. Primer design

Primers were designed to contain olignuceotide sequences which would either amplify all three polyomaviruses (consensus primers) or specifically only amplify one of the polyomaviruses (specific primers). In the design of these primers, consideration was taken to allow them to be used in any combination in multiplex or specific PCRs. Therefore they were designed so that they would have similar optima in a PCR.

All the primers were designed to lie within the highly conserved large T region. The primer sequences were all 20-24 bases long. When primers were required to bind with more than one polyomavirus, particular attention was given to ensure that the 3' terminal end was conserved to all polyomaviruses. The primers had a G+C content of between 41-55%, with the one exception of SV3. This primer was designed to specifically bind SV40 and lies within an A+T rich part of the genome. Long stretches of any one base were avoided since this could lead to non-specific annealing of the primer. Primer sequences were checked for self-complementarity to avoid internal secondary structures like hairpin loops. Primer pairs were checked for complementarity to avoid primer dimers.

The melting temperature (Tm) was calculated for each primer by the following formula:

$$Tm = 2^0C \times (A+T) + 4^0C \times (C+G) \quad \text{(Thein \& Wallace 1986)}$$

Primers to be used in the same PCR mixture were designed to have a similar Tm to help give similar optima in the PCR including melt and anneal temperature. The specific primers used in multiplex PCR were designed so that the amplified products could be differentiated on agarose gel. Therefore the primers were sited at specific points on the genome to give different length PCR amplification products and/or include a restriction enzyme site.

3.4. Multiplex semi-nested PCR

3.4.1. Design of semi-nested PCR

A multiplex PCR was developed to amplify JCV, BKV and SV40 DNA in a semi-nested PCR. Consensus primers CP3 and CP4 were designed which amplified a PCR product of 606-610 base pairs (depending on the virus) from the large T section of the polyomaviral genome. This was followed by a second round amplification stage using CP3 and three specific primers CP4-J (JCV-specific), CP4-B (BKV-specific) and CP4-S (SV40-specific). The three specific primers were sited at different sites on the genome, therefore the second round amplification would give different size PCR products for each polyomavirus (Fig 3.2). The size of the amplification products depended on the distance the specific primer binding site was from CP3. The product sizes were 347, 371 and 488 base pairs for JCV, SV40 and BKV respectively. Restriction fragment length polymorphisms (RFLP) were used to validate the specificity of the amplified PCR product. First round products amplified from JCV and BKVs were both cut into 2 fragments by the restriction endonuclease Hind III.

The size of these fragments were 298-309 base pairs and it was not possible to distinguish between them on an agarose gel after electrophoreses. The first round product generated from SV40 DNA did not contain a Hind III cleavage site. Digestion with the restriction endonuclease Bam HI gave fragments of 107 and 240 base pairs from a JCV second round amplified product, but did not cut BKV or SV40.

3.4.2. Detection of polyomaviral DNA in clinical samples

In order to obtain polyomaviral DNA, a series of urine samples were collected from patients undergoing therapy for haematological malignancies. DNA was extracted from the urine by the heat extraction method and a series of tenfold dilutions prepared for each sample. Each dilution was screened for polyomavirus sequences by PCR using the consensus primers CP3 and CP4. All PCRs had final concentrations of 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris (pH 8.3), 200 μM dNTP, 1.25 unit of AmpliTaq DNA polymerase (Perkin Elmer) and 100 ng of each primer. The first round reaction was carried out in a total volume of 50μl. Initial denaturation at 94^{o}C for 4 minutes followed by 35 cycles of 94^{o}C for 1 minute (denaturation), 48^{o}C for 1 minute (annealing), and 72^{o}C for 1 minute (extension), with an additional 7 minute extension. In two of the samples, P11 and P49, it was possible to visualise on an agarose gel stained with ethidium bromide products of approximately 600 base pairs which would be consistent with polyomaviral DNA being present in the samples. Both these samples were cut into two fragments by the restriction endonuclease Hind III. No amplified DNA could be visualised in any of the other samples and, since the aim was just to obtain polyomaviral DNA, no attempt was made to look for possible PCR inhibition in urine at this stage.

Second round PCR (semi-nest) was carried out on P11, P49 and P50 (a sample which showed no visualised amplified DNA in the first round) using the consensus primer CP3 and one of the specific primers. The reaction was carried out in a total volume of 25μl for 25 cycles of 94^{o}C for 1 minute (denaturation), 48^{o}C for 1 minute (annealing), and 72^{o}C for 1 minute (extension), with an additional 7 minute extension. In sample P11 products of the appropriate size to represent JCV (347bp) and BKV (488) DNA amplification were visualised on agarose gel (Fig. 3.3.), indicating the presence of both JCV and BKV. This was confirmed with the restriction enzyme Bam HI which digested the PCR products to give fragments of 107, 240 and 488 base pairs. The JCV DNA was cleaved into two fragments (107 and 240bp) while the BKV DNA was uncleaved (488bp). BKV DNA was amplified from sample P49, with only one product size on the agarose gel not cut by Bam HI. Sample P50 showed no amplified polyomaviral DNA after completion of the 25 cycles. A multiplex PCR which contained the specific primers CP4-J and CP-B detected both JCV and BKV DNA although there was a log drop in the sensitivity in BKV DNA detection (Table 3.2).

3.5. Specific nested PCR

3.5.1. Design of the nested PCRs

Three specific nested PCRs were developed to detect JCV, BKV and SV40 DNA individually or together as a multiplex PCR. All primers were designed to amplify DNA located in the large T section of the polyomaviral genome. These primer sequences were designed to be used in individual PCRs. However, they were selected so that the amplified products after nested PCR were of different size and so they could be differentiated on an agarose gel in a multiplex PCR.

For the detection of JCV DNA the oligonuceotide sequences were selected so that the nested PCR product would be 150 base pairs long (Fig 3.4). The primer JC2 was the same sequence as CP4-J (Table 3.3) but was renamed for simplicity on its publication (Perrons *et al* 1996). The sequence of JC3 was a modified version of a published oligonucleotide PEP-1 (Arthur *et al* 1989). The oligonucleotide sequences for BKV DNA amplification were designed and positioned to give a nested product of 301 base pairs long. The inner primer pairs were located in a region of high homology between the JCV and BKV genome and there was a problem finding a specific sequence for both of the inner primers. Therefore, for one inner primer BK3, it was elected to use a conserved sequence. Although BK3 had the same sequence as JC3, the other nested primer BK4 was specific for the BKV virus. Again, the oligonucleotide sequence CP4-B was renamed to BK2 for publication. The oligonucleotide sequence designed to amplify SV40 DNA gave a nested product of 107 base pairs long. All the primers were screened against the library file JCBKSV and data bases for specificity and were aligned against each other.

The restriction endonuclease Bam HI was used to validate the specificity of the nested amplified PCR product. This enzyme cleaved JCV into two fragments, 55 and 95 base pairs long. The nested PCR products of BKV and SV40 did not contain a Bam HI cleavage site, therefore their PCR products of 301 and 107 base pairs respectively remained uncut. The PCRs were designed so that further confirmation could be achieved using the restriction endonuclease Hind III which cut the BKV nested amplified product, but not the JCV or BKV product.

3.5.2. Detection of JCV and BKV DNA in urine by nested PCR

The first PCRs using the specific primers designed to detect JC and BK viruses were performed using the urine samples P11 and P45. All PCRs had final concentrations of 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris (pH 8.3), 200 μM dNTP, 1.25 unit of AmpliTaq DNA polymerase (Perkin Elmer) and 100 ng of each primer. The first round of both these PCRs was carried out in a total volume of 50μl reaction mixture containing the outer primers. An initial denaturation at 94^{o}C for 4 minutes followed by 35 cycles of 94^{o}C for 1 minute (denaturation), 52^{o}C for 1 minute (annealing), and 72^{o}C for 1 minute (extension), with an additional 7 minute extension. After completion of the 35 cycles 1μl of the first round product was transferred to a fresh PCR mixture containing the "inner" (nested) primers. The second round PCR reaction was carried out in a total volume of 25μl with the same conditions as in the first amplification, except the primer concentration was doubled and the initial 4 minutes denaturation at 94^{o}C was omitted. Twenty five cycles of PCR were found to be sufficient for the detection of 10μl of the second round products by electrophoresis on a 3% ethidium bromide-stained agarose gel. In the BKV PCR the anneal temperature of 52^{o}C resulted in non-specific binding, therefore the anneal was increased to 56^{o}C.

The JCV primers amplified DNA of the appropriate size in the urine samples P11 and P49, which was confirmed by cutting the PCR products into two fragments with the endonuclease Bam HI. The PCRs were performed on a tenfold dilution series of P11 and P49 and the end point titration was 1×10^{-6} and 1×10^{-8} respectively.

3.5.3. Controls

Since the amount of clinical material for controls was limited, and because there was no detectable SV40 in the samples available, controls were prepared from tissue culture supernatants or first round PCR products. Tissue culture of supernatants of BKV and SV40 were obtained from Department of Virology, Royal Postgraduate Medical School. The DNA was extracted by the heat method and 10-fold dilutions were prepared in H_2O containing 50µg/ml salmon sperm DNA (negative carrier). The nested PCRs amplified BKV and SV40 from these supernatants with an end-point of $1x10^{-5}$ and $1x10^{-8}$ respectively. It was decided to use end-points for the positive controls and, to determine any change in sensitivity the dilution on either side of the end-points were also put into each reaction. Aliquots of these dilutions were stored at -20°C, thawed and put into each PCR along with negative controls. At the time of setting up this project we were unable to get any non-clinical JCV DNA because of the difficulty of growing it in tissue culture. Therefore, a first round PCR product amplified from urine (described in section 3.5.2) was diluted in salmon sperm DNA diluent. Using nested PCR it was possible to detect JCV DNA to an end point of $1x10^{-11}$ and this dilution and the ones on either side of it were used as controls. The controls were stored at -20°C and after twenty runs a new PCR product was diluted and amplified by PCR along side the old control. The controls remained stable for these 20 runs, but to avoid any degradation of the PCR product a new control was always prepared every 20 runs.

3.5.4. Specificity of the primers

The three sets of primers were designed only to amplify DNA from only one type of polyomavirus. Computer analysis had been used to check the amount of mismatch

between the primer sequences, but to confirm this a checker board experiment was carried out. A panel consisting of dilutions from first round PCR products (JCV) or tissue culture supernatants (BKV and SV40) was prepared. Using the individual primers this panel was subjected to the three nested PCRs and second round products detected on an ethidium bromide-stained agarose gel (Table 3.4). All the primer sets amplified DNA from the controls they were designed for, but not from the other controls in the panel. No BKV DNA was detected by the BKV specific primers in the neat tissue culture supernatant, identified as inhibition by a reaction in the first ten-fold dilution. There was possibility of inhibition by the tissue culture media, but this was avoided by using a 10 fold dilution stage.

3.6. Discussion

At the time of design of these PCRs it was not known what viral DNA the samples would contain and there was the possibility of the need to test for mixed samples. Therefore it was important to establish conditions that would enable the amplification of more than one target. Although in the first studies it was decided to keep the specific sets of primers separately at first, until each set had established its ability to amplify the appropriate section of DNA.

In the semi-nested PCR there was a drop in sensitivity when it was used as a multiplex PCR to detect both JCV and BKV in a single sample (Table 3.2). The BKV was detected at a 10-fold greater concentration when amplified in the absence of any specific JCV primers. This was probably due to enzyme becoming rate limiting. In some multiplex PCRs the amplicons which anneal more efficiently with a primer will preferentially bind free enzymes and will be amplified to a greater degree than other amplicons. In this multiplex both JCV and BKV DNA were amplified to

approximately the same levels, indicating they were using up the enzyme activity at equal rates and the loss of sensitivity was probably due to enzyme exhaustion. Therefore on future work on urine studies the enzyme concentration should be increased when multiplexing.

Overall the semi-nested PCR was less sensitive than the individual JCV and BKV nested PCR and therefore it was necessary to put in considerable effect in to optimising it. Since the aim of the first part of the study was to using single target PCR it was decided not to optimise the semi-nest at this stage. If necessary the three virus specific nested PCRs could be combined at a later stage.

Table 3.1. Alignment of polyomavirus nucleotide sequences for primer design in the semi-nested multiplex PCR (5'→3')

```
Consensus Primers
_____________________________________________________

Nucleotide position       4203
PAPPLYCG1                 GTTCTATTACTAAACACAGC
SV40XX                    GTTCTATTACTAAACACAGC
PABKVDN                   GTTCTATTACTAAATACAGC
Alignment                 ************** *****
Primer CP3                GTTCTATTACTAAACACAGC

Nucleotide position       4809
PAPPLYCG1                 CCAAAATCAGGCTGATGAGC
SV40XX                    CCAAAGTCAGGTTGATGAGC
PABKVDN                   CCAAAATCAGGCTGATGAGC
Alignment                 ***** ***** ********
Primer CP4                CCAAAATCAGGCTGATGAGC

Specific primer for JCV
_____________________________________________________

Nucleotide position       4549
PAPPLYCG1                 GAACTTTCTCCCAGCAATGA
SV40XX                    T---TATGTCACACCACAGA
PABKVDN                   TTATTTGGACCCACCATTGC
Alignment                     *    * ** **  *
Primer CP4-J              GAACTTTCTCCCAGCAATGA

Specific primer for SV40
_____________________________________________________

Nucleotide position       4583
PAPPLYCG1                 GTTAAG-TCACACCCAAACC
SV40XX                    ACAAAGATCAAGTCCAAACC
PABKVDN                   AGTTAGGTCTAAGCCAAACC
Alignment                     ** **    *******
Primer CP4-S              ACAAAGATCAAGTCCAAACC

Specific primer for BKV
_____________________________________________________

Nucleotide position       4691
PAPPLYCG1                 ATGCACATTAAACAGGGGCA
SV40XX                    AAGCACAGCAAGCATATGCA
PABKVDN                   TGACATAGCATGCAAGGGCA
Alignment                    ** *  *  **   ***
Primer CP4-B              TGACATAGCATGCAAGGGCA
```

Table 3.2. Polyomaviral detected in urine using the semi-nested PCR

Urine sample	Primers used in semi-nest		
	CP3/CP4-J	CP3/CP4-B	CP3/CP4-J/CP-B
P11	JCV DNA detected (endpoint 1 x 10^{-4})	BKV DNA detected (endpoint 1 x 10^{-5})	JCV DNA detected (endpoint 1 x 10^{-4}) BKV DNA detected (endpoint 1 x 10^{-4})
P49	JCV DNA not detected	BKV DNA detected (endpoint 1 x 10^{-7})	BKV DNA detected (endpoint 1 x 10^{-7})
P50	JCV DNA not detected	BKV DNA not detected	JCV and BKV DNA not detected

Table 3.3. PCR oligonucleotide primers for specific JCV, BKV, and SV40

Virus	Oligonucleotide	Sequence (5'→3')	Nucleotide position
JC Virus	JC1	CTGAGGAATGCATGCAGATCT	4227-4247
	JC2	TCATTGCTGGGAGAAAGTTC	4549-4530
	JC3*	AAGTCTTTAGGGTCTTCTACCT	4275-4254
	JC4	ATGGGAATCCTGGTGGAATACA	4403-4382
BK virus	BK1	AAGAAACTGGTGTAGATCAGAG	4366-4387
	BK2	TGCCCTTGCGTGCTATGTCA	4829-4810
	BK3*	AAGTCTTTAGGGTCTTCTACCT	4391-4412
	BK4	CTGCAATGGTGGGTCCAAAT	4691-4672
Simian virus 40	SV1	AGCCTCATCATCACTAGATGG	4470-4490
	SV2	TGTGGTTTGGACTTGATCTTTG	4727-4706
	SV3	GAATCAGTAGTTTAACACATTATA	4592-4615
	SV4	ACCTTACTTCTGTGGTGTGAC	4698-4678

* Modified from PEP-1 (Arthur *et al* 1989).

Table 3.4. Polyomaviral DNA detected in a panel of controls using individual nested PCR

Control	Dilution	Detection of polyomaviral DNA by specific nested primers		
		JCV	BKV	SV40
BKV tissue culture supernatant	neat	not detected	not detected	not detected
	1×10^{-1}	not detected	detected	not detected
SV40 tissue culture supernatant	neat	not detected	not detected	detected
	1×10^{-1}	not detected	not detected	detected
JCV first round PCR product	1×10^{-6}	detected	not detected	not detected
	1×10^{-7}	detected	not detected	not detected

Fig. 3.1. Percentage nucleotide homology between JCV, BKV and SV40

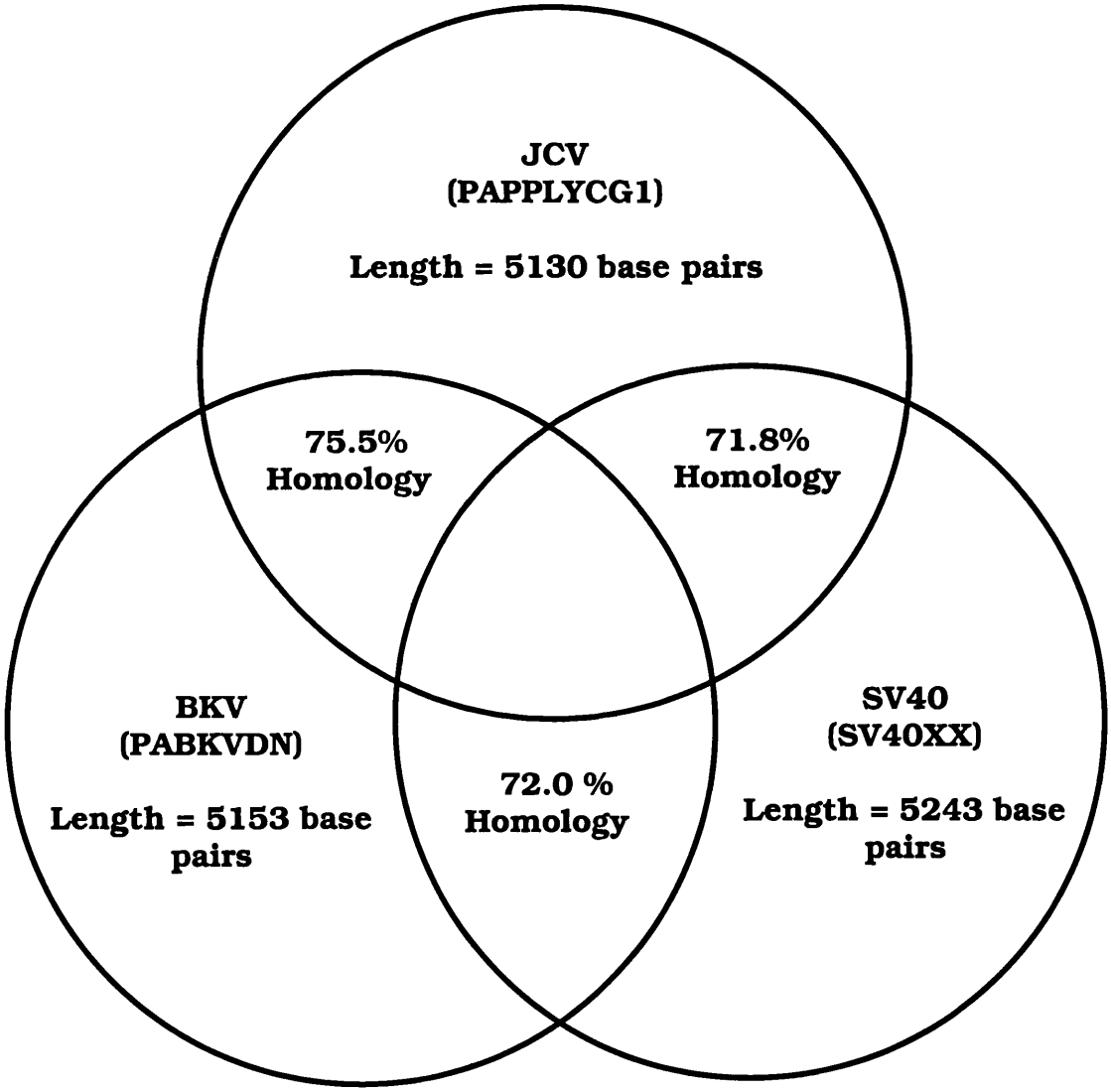
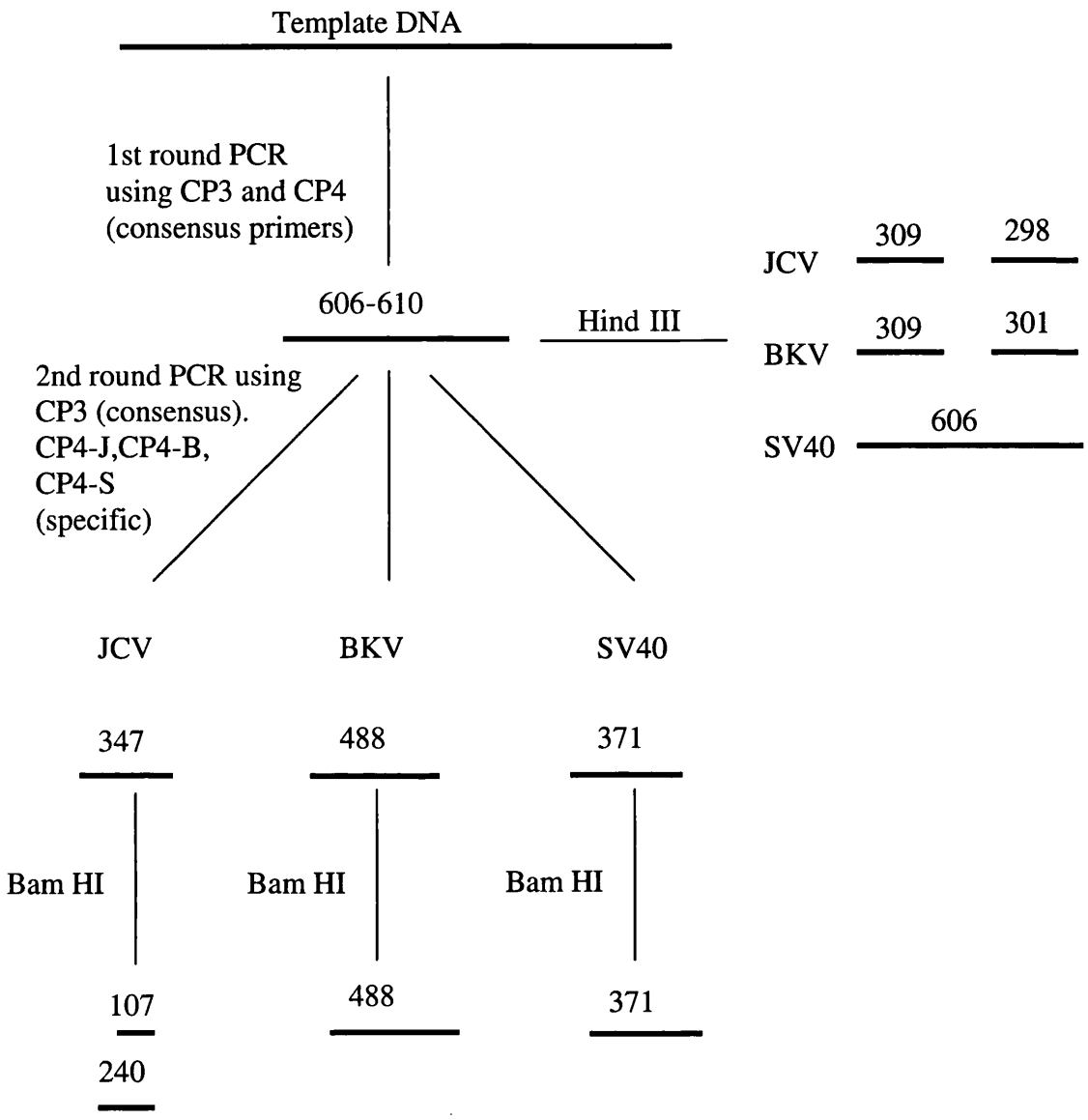


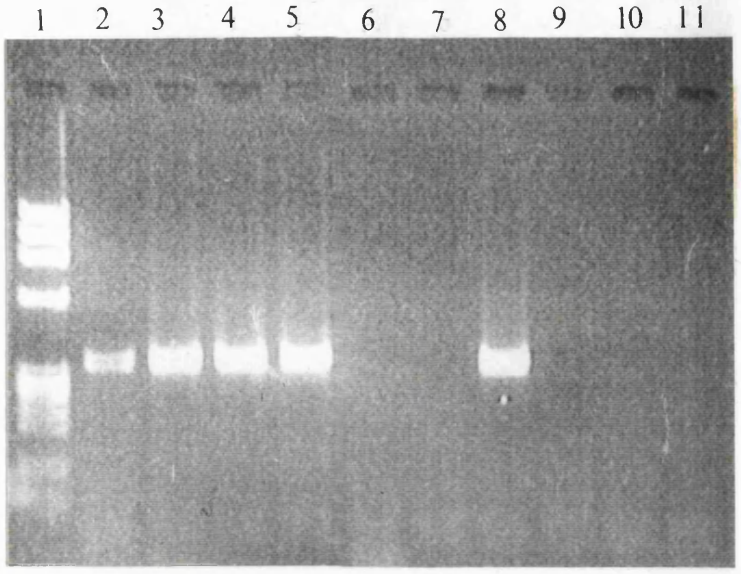
Fig. 3.2. Diagrammatic scheme of multiplex semi-nested PCR



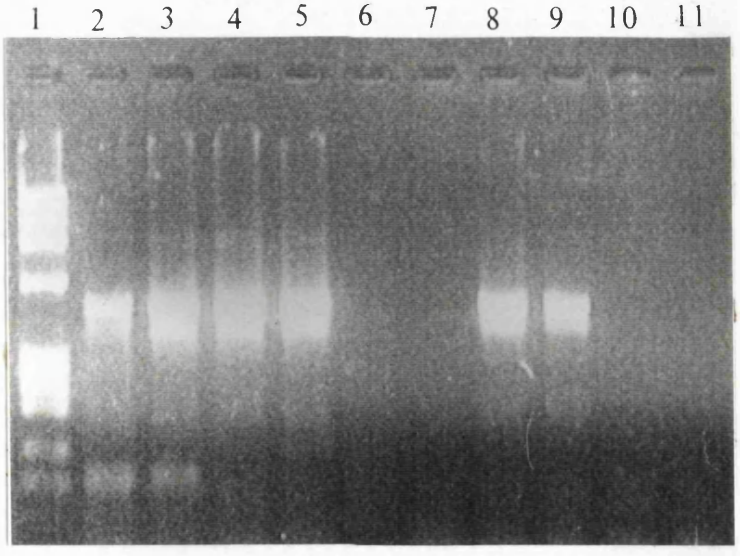
The numbers represent the length of the nucleotides

Fig. 3.3. JCV and BKV products amplified by the semi-nested PCR

Second round PCR products amplified by CP3 and CP4-J



Second round PCR products amplified by CP3 and CP4-B



Lanes 1: Molecular weight marker ∅X174 cut with Hae III

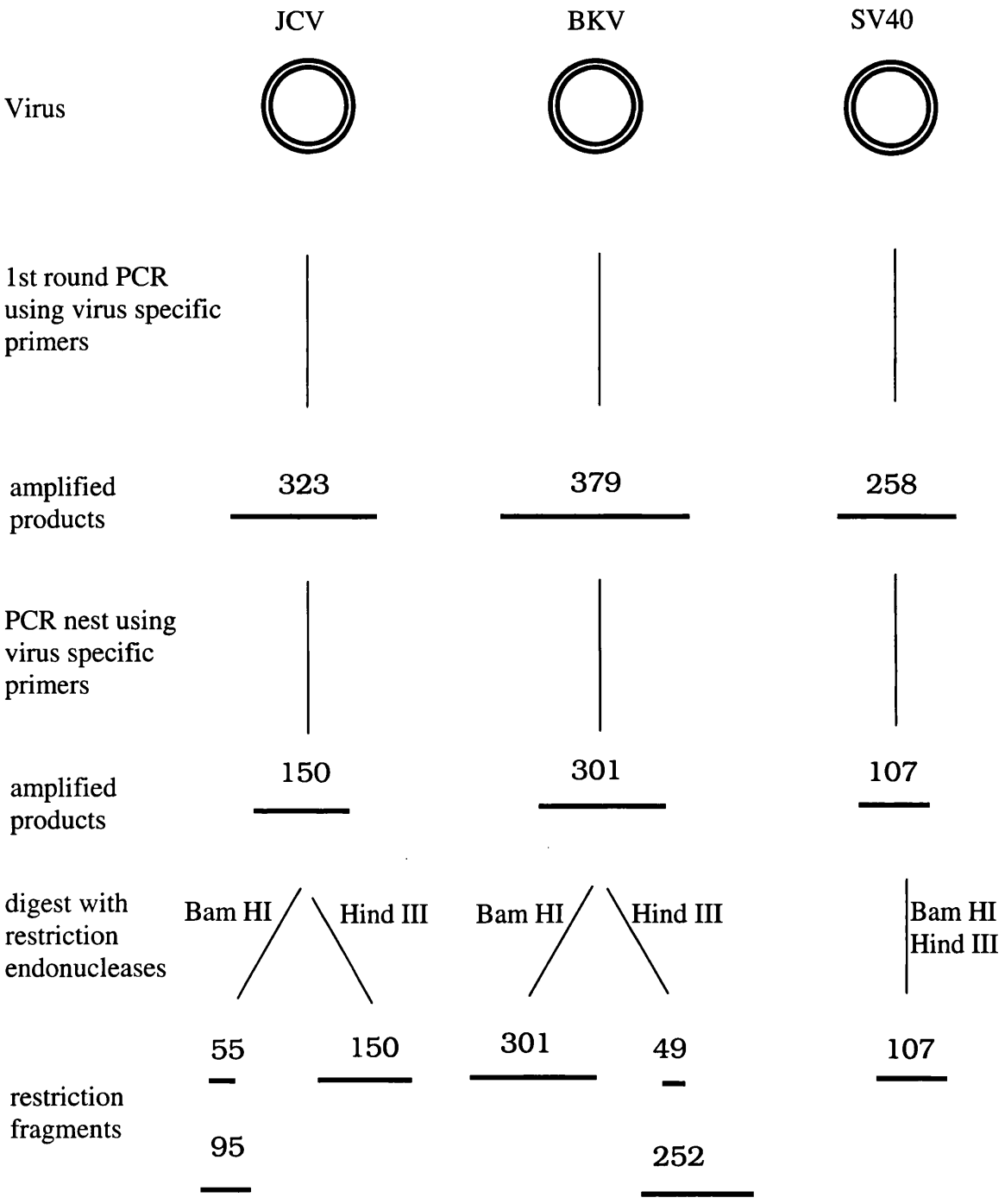
Lanes 2-5: Ten fold dilution series of P11 urine (neat to 1 x 10^{-3})

Lanes 6/7: Negative water controls

Lanes 8-9 : Ten fold dilution series of P11 urine (1 x 10^{-4} to 1 x 10^{-5})

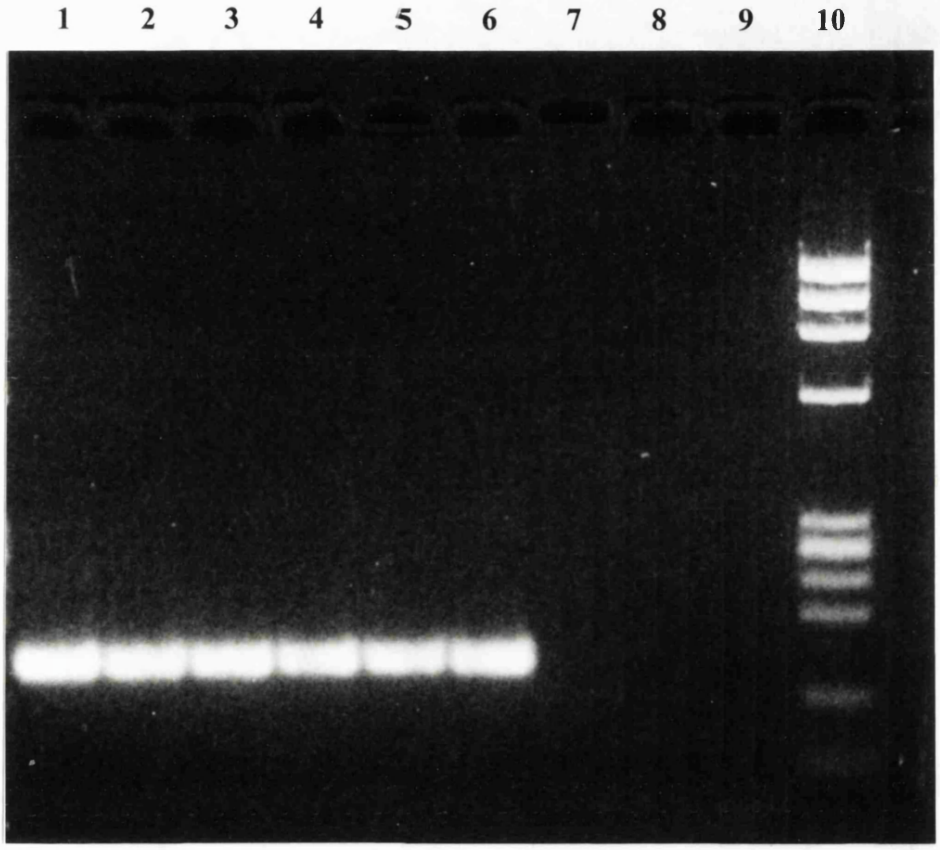
Lanes 10/11 : Negative water controls

Fig. 3.4. Diagrammatic scheme of the three polyomaviral specific nested PCRs



The numbers represent the length of the nucleotides

Fig. 3.5. JCV products amplified by nested PCR using the JC1-4 primer set



Lanes 1-6: Ten fold dilution series of P11 urine containing detectable JCV DNA (1×10^{-1} to 1×10^{-6})

Lane 7: 1×10^{-7} dilution of P11 urine containing no detectable JCV DNA

Lanes 8/9: Negative water controls

Lane 10: Molecular weight marker ∅X174 cut with Hae III

Chapter 4: Four linked studies on the detection of polyomavirus in clinical samples from PML patients and non-PML patients

4.1. Introduction

The studies in the previous chapter looked at the development of the PCR methods for the detection of polyomaviruses. From these studies it was decided to use the specific nested PCRs for JCV, BKV and SV40. These were designed so they could be used in a multiplex PCR if needed. These early studies were carried out using viral DNA extracted from either tissue culture supernatant or urine samples. In the studies in this chapter the aim was to determine how these PCRs performed on clinical samples taken from the central nervous system (CNS).

There had been reports of JCV, BKV and SV40 present in the CNS (Telenti *et al* 1990; Dorries *et al* 1987; Weiner *et al* 1972) and to a varying degree all these viruses have been associated with disease. JCV in particular had been implicated as the aetiological agent of PML. One of the major aims was to look at the proposal that the presence of JCV DNA in the CSF could be used as an indicator that there was JC virus present in the brain. Therefore, the first study in this section was to look at the CSF sample, how it had been treated and what part of the fractionated CSF was most appropriate to use for amplification of JCV specific sequences. The second of these studies was an investigation into whether BKV or JCV was present in the CNS and if there was any association with PML. The third of these studies was to set up a small study to look at the association between JCV and PML. The aim was to look at archival material which had some clinical data before applying for funding for a larger study. This was a blind

study to look at the presence of JC virus in CSF and then compare it with the diagnosis made by histology, neuroimaging and clinical symptoms. This study also gave an opportunity to look for the presence of both BKV and SV40 DNA in CSF. Finally, the last of these studies was to look at the prevalence of CMV in the CSF of patients with PML. This was to determine the level of concomitant CMV in these patients and whether this indicated any interaction between it and JCV. The results of these sub-studies will be discussed in each section with a general discussion at the end of this chapter.

4.2. Sub-study one: Detection of JCV DNA in whole or fractionated CSF

4.2.1. Samples and methods

The original method used to extract JCV DNA from CSF was to boil the sample for 5 minutes. Prospective samples were separated on arrival into pellet and supernatant and an aliquot of the whole CSF. Therefore it was necessary to evaluate which part of the CSF was the most appropriate to test for the detection of JCV sequences. Sufficient CSF was available from 37 patients to evaluate which of the cell pellet, supernatant or unfractionated CSF would be most suitable for diagnostic PCR. All the patients were immunosuppressed with acute neurological symptoms and a CSF sample had been taken for virological analysis. Twelve of these patients were diagnosed as having PML on the basis of MRI and clinical symptoms. One of these patients had a further CSF sample taken 2 months later.

DNA was extracted from the analyte by the heat extraction method as described in chapter 2. All three types of samples were analysed separately by nested JCV PCR and the input into the PCR mixture was 10μl. The CSF pellets had been resuspended into

sterile water to give 5% of the original volume, and so it was only possible to test those samples where 200μl or more had been available for initial centrifugation.

4.2.2. Results

No JCV sequences were detected in any of the CSF samples taken from the patients with no clinical diagnosis of PML (Table 4.1). While 10 of the 12 patients clinically diagnosed with PML had JCV DNA detected in at least one of their CSF samples, the ability to detect JC virus was greatly influenced by the treatment of the CSF. There was no difference in the sensitivity of JCV DNA in unfractionated or supernatant CSF and by testing these two types of samples JCV DNA was only detected in 7 of 12 (58%) of the patients clinically diagnosed with PML. Interestingly, four samples from the patients with a clinical diagnosis of PML contained detectable JCV-specific DNA in the CSF cell pellet but not in the supernatant or unfractionated CSF samples. Also in 3 patients JCV was only detected in the supernatant and unfractionated samples and not in any pelleted material. Therefore by testing both the supernatant and pellet it was possible to detect 10 of 12 (83%) patients clinically diagnosed with PML. These results indicate that detection was on the edge of clinical sensitivity and therefore necessary to test both samples to maintain this sensitivity.

In patient 6 JCV DNA was originally only detected in the CSF pellet, but a further sample was taken two months later and treated in exactly the same way as the first. In this later sample JCV DNA was also detected in the unfractionated CSF, supernatant and cell pellet material. To try and improve the sensitivity the original unfractionated CSF and supernatant samples from patient 1 were ethanol precipitated and resuspended in one tenth the original volume. Therefore possible DNA from the equivalent of 100μl of each sample was added in 10μl to the PCR mixture. It was not possible to detect JCV-specific

DNA in these 2 samples even after this concentration step. The supernatant from the first CSF sample taken from patient 6 was spiked with JCV positive and showed no inhibitory effect.

4.3. Sub-study two: Analysis of CSF and brain tissue for the prevalence of BKV and SV40 in immunocompromised patients

4.3.1. Samples and methods

There had been conflicting evidence on the role of BKV and SV40 in CNS disease. Therefore the aim of this study was to determine if BKV or SV40 were present in the CNS and if so, whether there was any correlation with PML. CSF samples were obtained from 90 immunocompromised patients who were undergoing surveillance because they presented with neurological symptoms. Twenty three of these patients had been diagnosed with PML on the basis of clinical symptoms and neuroimaging. The DNA was extracted by boiling and put directly into the DNA amplification stage. Twenty three samples of brain tissue (frontal lobe) were taken at post-mortem for histological examination. Four were diagnosed to have PML by a histological examination. The DNA was extracted by the sodium dodeyl sulphate (SDS) lysis method and amplification of human pyruvate dehydrogenase sequences was achieved in all samples. The extract (1μl) was put directly into the BKV or SV40-specific nested PCR at a dilution which would remove any inhibitory effect of the PCR by SDS.

4.3.2. Results

No BKV or SV40 sequences could be amplified by the specific nested PCRs from any of the 90 CSF samples or the 23 brain tissue samples taken at post-mortem (Table 4.2). These results suggest there was no evidence for the association of BKV or SV40 with

CNS disease in this group of patients. However it could have been possible that the virus was present in the brain at sites not sampled at autopsy or at levels below our threshold for detection.

4.4. Sub-study three: A study using PCR to detect polyomaviruses in CSF from a clinical cohort of patients

4.4.1. Samples and methods

This study was carried out in collaboration with Professor Joseph Berger while he was working at the University of Miami School of Medicine. The aim of the study was to evaluate the three polyomaviral nested PCRs on clinical samples. The samples provided by Professor Berger were CSFs taken from patients with neurological symptoms. The clinical details of these patients were not released until the study had been completed, but it was known that some of the samples had come from patients diagnosed with PML or suspected PML. The 16 CSF samples, which had been frozen as whole unfractionated CSF and stored at -20^{0}C, were transported to London on dry ice by courier and immediately stored unthawed. At the time we analysed these samples we believed they were supernatants of spun CSF, therefore the sample was not spun. The DNA was extracted by the heat method and placed on ice. Using the specific nested PCRs, 10μl of each CSF was tested for the presence of JCV, BKV and SV40-specific sequences.

4.4.2. Results.

The samples were then decoded and the patients put into the following groups by Professor Berger according to their clinical data.

Confirmed PML: Patients with a biopsy or autopsy confirmed diagnosis revealing the characteristic neuropathological hallmarks of PML with *in situ* hybridisation showing JC virus.

Suspected PML: Patients with focal neurological findings and an MRI with hyperintense lesions of the white matter on T2WI without mass effect or contrast enhancement. No other aetiologies were discerned despite intensive investigation.

No PML: Patients with no known neuropathological characteristics of PML.

The CSF samples were taken from 14 patients, of which two from the 'confirmed PML' group had consecutive samples taken at 7 and 17 weeks later.

No BKV or SV40 sequences were detected in any of the CSF samples (Table 4.3). JCV DNA was detected in 5 CSF samples, 4 of these from patients with confirmed PML and one from a patient with suspected PML. Where there were consecutive CSF samples from patients with confirmed PML, JCV DNA was not detected in the early sample but in both cases detected in the second sample.

4.5. Sub-study four: Prevalence of cytomegalovirus (CMV) in CSF of PML patients

4.5.1. Samples and methods

Studies based on fibroblast cultures have indicated that the replication of cytomegalovirus (CMV) may lead to the reactivation of latent JCV (Heibbronn, 1993). Therefore, it was decided to look at PML patients to see if there was concomitant CMV in the CSF. A series of 28 CSF supernatant samples were available from patients with PML in which JCV DNA had been detected in every case. Of the PML patients in which JCV DNA had not been detected in their CSF, there was only sufficient sample available

from 3 for further analysis. The DNA was extracted by boiling and the extract (10μl) was put directly into CMV specific nested PCR.

4.5.2. Results

Attempts to amplify CMV-specific sequences in these samples by nested PCR showed that only 2 of the samples contained both viruses. (Table 4.4). No CMV DNA was amplified from 3 CSF samples. Therefore 2 of 28 (7%) patients with a clinical diagnosis of PML had concomitant CMV in the CSF, suggesting that if CMV does play a role in reactivation of JCV it is unlikely to happen in the CNS.

4.6. Discussion

In the first study there was no apparent difference between the ability to detect JCV DNA in unfractionated and supernatant CSF. The ability to detect JC virus increased if the CSF sample was centrifuged and both the supernatant and resuspended pellet was tested. The pellet was resuspended in 5% of the original volume and the attempts to amplify JCV sequences gave mixed results. In some samples it was possible to amplify JCV DNA in the resuspended pellet but not in the whole or supernatant CSF. This could have been caused by some concentration effect of cell free virus, although unlikely because the sample was only centrifuged at 15,000g for 5 minutes. Sometimes the pellet was visible, but mostly it was not and one could only speculate as to the exact composition of the pellet. If CSF contained cells it would be possible that they would be pelleted with centrifugation along with any virus associated with them.

There were samples where the viral DNA had been amplified in whole and supernatant CSF but not in the resuspended pellet. This could be due to an inhibitory effect on the PCR caused by material being pelleted by centrifugation. For example, blood could

contaminate the sample during the spinal tap and blood contains porphyrin compounds derived from haemoglobin which are highly inhibitory to PCR (Higuchi 1989). It was concluded that the highest sensitivity (83%) could only be achieved by testing the cell pellet along with either the supernatant or whole CSF (Fig 4.1).

In the second study no BKV or SV40 sequences were detected in the CSF from 28 patients with a clinical diagnosis of PML or in any of the CSF samples taken from the patients in the control group (n=67). At the time of these studies it was still believed that the definitive diagnosis of PML was by histological methods on brain tissue. Nine of the CSF samples had been taken from PML patients who had been diagnosed by brain biopsy or at autopsy. Therefore these findings show no evidence of either BKV or SV40 being present in CSF, or any correlation with the disease PML. Whatever the correlation was with disease, there was still the question whether or not BKV or SV40 were latent in brain tissue. These studies had looked for 2 polyomaviruses in 23 brain tissue samples, 4 of which were taken from patients diagnosed with PML. That no BKV or SV40 sequences were amplified from any of these samples, does not rule out the possibility of low level latent infection in the brain. This was because the sampling was specifically aimed for PML diagnosis and only small amounts of frontal lobe tissue were analysed. It could still be possible for one or other of these polyomaviruses to be present in the brain at sites not sampled or at levels below the threshold for detection by the methods employed.

In the third study the pilot study carried out on the samples taken from the 14 patients from Miami showed a correlation with the presence of JC virus in CSF and the disease PML. In these early studies there was a grey area in the diagnosis of PML in the absence of brain biopsy or autopsy material. Therefore it was difficult to interpret the patient group "suspected PML" and the sensitivity in this study was estimated at 50-80%.

However there was no JCV DNA detected in the control group. This group, although small (n=4), gave the initiative to set up a larger study (this will be presented in the next chapter).

In the patients where consecutive samples were taken, JCV DNA was not detected in the first sample but was amplified in the second sample. This was also shown in patient 6 from the first sub-study and may be due to an increase in viral load due to the progress of disease. Therefore the detection of JCV DNA in CSF could depend on the stage in the progress of disease from when the sample is taken.

In the fourth study it was shown that only 7% of CSF obtained from patients diagnosed with PML had both detectable JCV and CMV. Therefore no further studies were carried out to look at any interaction between these viruses.

Table 4.1. Detection of JCV DNA in whole or fractionated CSF

Patient group	Patient	Whole CSF	CSF supernatant	CSF pellet
Diagnosed PML (n=12)	1	-	-	-
	2	-	-	-
	3	-	-	+
	4	-	-	+
	5	-	-	+
	6a	-	-	+
	6b*	+	+	+
	7	+	+	+
	8	+	+	+
	9	+	+	+
	10	+	+	-
	11	+	+	-
	12	+	+	-
Not diagnosed PML (n=25)	13-37	-	-	-

* samples 6a and 6b was taken from the same patient, the later taken two months later.

Table 4.2. Detection of BKV and SV40 DNA in clinical samples from immunocompromised patients

Patient	BKV DNA detected		SV40 DNA detected	
	CSF	Brain	CSF	Brain
Diagnosed PML	0/23	0/4	0/23	0/4
Not diagnosed PML	0/67	0/19	0/67	0/18

Table 4.3. Prevalence of polyomaviruses in CSF

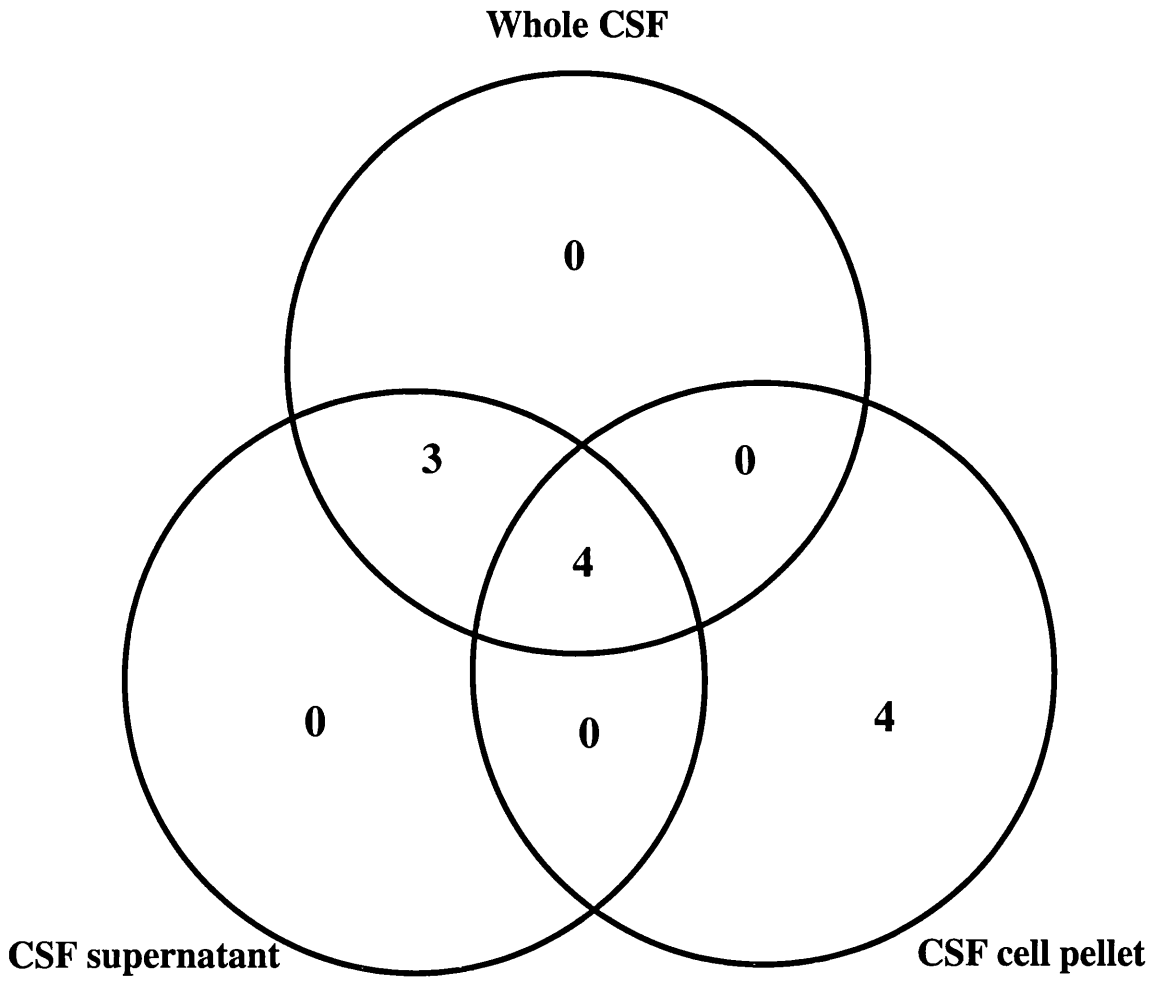
Type of patient	Polyomavirus detected in CSF		
	JCV DNA detected	BKV DNA detected	SV40 DNA detected
Confirmed PML patients	2/5*	0/5	0/5
Suspected PML patients	1/5	0/5	0/5
Non PML patients	0/4	0/4	0/4

* In two of these patients there was a second CSF sample taken at 7 and 17 weeks later. JCV DNA was not detected in the early samples but in both cases detected in the second sample.

Table 4.4. Prevalence of cytomegalovirus (CMV) in CSF samples from PML patients

PML patient	CMV DNA detected	CMV DNA not detected
JCV detected in CSF (n=28)	2	26
JCV not detected in CSF (n=3)	0	3

Fig. 4.1. Venn diagram showing the relationship between the detection of JCV DNA in whole and fractionated CSF



Chapter 5: Detection of polyomaviral DNA in clinical samples from immunocompromised patients: correlation with clinical disease

5.1. Introduction

In this study polymerase chain reaction (PCR) amplification was used for detection of JCV DNA in clinical samples in order to determine if such a molecular approach would be useful in the laboratory diagnosis of PML. Also the prevalence of JCV CNS infections in immunosuppressed patients with neurological symptoms was estimated. The main aim was to determine if there was a correlation between these CNS infections and PML. This investigation was funded by the Medical Research Council UK and completed in 1996. There have been a number of similar studies which agree with our findings. The patients in the study were attending one of 6 London hospitals and all the samples were sent to University College London Medical School for virological analysis. The hospitals involved were University College London, Chelsea and Westminster, St Bartholomew's, Hammersmith, St George's and Guy's Hospitals

5.2. Patients

Cerebrospinal Fluid samples were available for study from 90 immunosuppressed patients who presented with acute neurological symptoms and signs. All patients underwent magnetic resonance imaging (MRI) and on the basis of clinical and diagnostic investigations were divided into 2 groups, one with a presumptive diagnosis of PML and the other without a diagnosis of PML. In addition there were urine and autopsy samples

from another 33 immunosuppressed patients who had not had any diagnosis of PML (Table 5.1).

Group 1 (PML): Twenty three patients (21 HIV-infected with CD4 counts 10-165 /mm^3) with focal white matter changes, without mass effect, showing high signal on T2 weighted images. The two HIV-uninfected patients were immunosuppressed because they were undergoing therapy for haematological malignancies. The other patients were immunosuppressed because of HIV disease. In this group 2 patients underwent ante-mortem brain biopsy to confirm the diagnosis and in 2 others there was post-mortem confirmation of PML. Urine and peripheral blood samples were available from 8 and 7 patients respectively.

Group 2 (controls): Sixty seven patients (62 HIV-infected with CD4 counts 1-200 /mm^3) in whom the MRI showed either abnormalities such as diffuse white matter changes or focal mass lesions, or was normal, or in whom there was an alternative neurological diagnosis such as toxoplasmosis or cryptococcosis made by routine CSF analysis. In 5 of these patients post-mortem histological examination of the brain failed to demonstrate PML. Urine and peripheral blood samples were available from 10 patients in this study group.

Additional control samples: Samples from 33 HIV-infected patients but without a clinical diagnosis of PML were available for analysis. These comprised frontal lobe tissue (n=14), and urine (n=19). The brain tissue was taken during the post-mortem examination and the absence of PML was confirmed by histology.

5.3. Prevalence of JC virus DNA in CSF

All CSF samples from patient group 1 and 2 were tested for the presence of JCV-specific sequences. The DNA was extracted by the boiling method and the DNA amplified by nested PCR. Since PML typically develops when the CD4 count is less than 200/mm^3 all the CD4 counts were recorded for both groups and one of the criteria of inclusion in patient group 2 was to have a CD4 count of 200/mm^3 or less. JC virus DNA was detected in 19 of 23 CSF samples from the PML group (Table 5.2). No JCV sequences could be detected in any of the CSF samples from the group not diagnosed with PML. The CD4 count of the patients of the two groups was similar in the two groups although the patients diagnosed with PML had a slightly higher median level. The CSF samples from patients with a clinical diagnosis of PML which did not contain detectable JCV sequences were spiked with a positive control and shown not to contain inhibitors of PCR.

5.4. Evaluation of the two DNA extraction methods on post-mortem brain tissue

All the brain tissue was extracted using the sodium dodecyl sulphate (SDS) lysis method and the lysed cell preparations were boiled and put directly into the DNA amplification stage. Additionally, 1 sample taken from a patient diagnosed with PML and 2 samples from patients not diagnosed with PML, were put though a phenol chloroform extraction prior to the amplification stage. The amount of the DNA preparation added to the PCR mixture was 10µl into a total volume of 50µl. The unamplified samples were diluted $\log_{10}$fold in water down to at least 1×10^{-4} or to the endpoint.

In the post-mortem samples from the patient with PML, JCV DNA sequences were detected down to a dilution of 1×10^{-6} after the SDS/phenol chloroform extraction

(Table 5.3). There was a tenfold increase in sensitivity if the SDS lysed cells were put directly into the PCR, although there appeared to be an inhibitory effect in the neat extract. In samples from the patients in whom PML was not diagnosed, no JC virus sequences were detected down to the 1 x 10^{-4} dilution with both extraction methods.

For operational convenience it was chosen to put the various dilutions of the lysed cells directly into the amplification reaction, but this was to be followed by a phenol chloroform extraction if brain tissue from PML patients was found not to have detectable JCV DNA present.

5.5. Prevalence of JC virus DNA in brain tissue

Traditionally the use of brain biopsy in the management of PML patients was usually limited to cases were it was necessary to differentiate PML from cerebral toxoplasmosis or other opportunistic infections. In this study brain biopsy was not performed on the patients infected with HIV, only on the 2 patients undergoing therapy for haematological malignancies. In addition 2 of the 21 patients with AIDS and a clinical diagnosis of PML also went on to have a post-mortem, at which brain tissue (frontal lobe) was taken. Thus, 4 samples of brain tissue were available from patients with PML. Five of the patients from group 2 also went on to have a post-mortem. Therefore with the additional 14 samples taken at the other post-mortems there were 19 brain samples from HIV-infected patients without a clinical diagnosis of PML available.

The DNA from all the 23 brain samples was extracted by the SDS/boil method and 1μl brain extract added to the PCR mixture. Also samples were tested at further tenfold and hundredfold dilution. The validity of these extractions was checked by using PCR to amplify human pyruvate dehydrogenase sequences. Amplification in all extracts confirmed that human DNA was extracted from all brain samples and detectable by

PCR. The nested PCR was used to determine if there was any evidence of JC virus DNA in these extracted samples. All the brain tissue from the patients with a diagnosis of PML contained detectable JCV-specific DNA. In 3 of these 4 patients JCV DNA was also detected in the CSF (Table 5.4). The fourth when spiked with JCV DNA was shown not to contain inhibitors of PCR. There was no evidence of JCV DNA in any of the post-mortem samples taken from patients who were considered to have died for reasons other than PML.

5.6. Detection of JC virus DNA in urine and peripheral blood

Only 18 urine samples were available from the 90 patients in groups 1 and 2. Therefore another 19 were collected from a group of patients who had AIDS, but who had no known neurological symptoms and signs. The DNA was extracted from the 37 urine samples by the heat extraction method and tested for the presence of JC virus by the nested PCR method. Because of the possible problem of urine presenting an inhibitory effect on the PCR, samples were tested at 3 dilution levels; neat, tenfold and hundredfold. Three of the 8 urine samples from patients with PML contained JCV-specific DNA but 3 of the 29 urine samples from immunosuppressed patients without a diagnosis of PML also contained detectable JCV sequences (Table 5.5).

The mononuclear cells from whole blood were separated by density-gradient centrifugation using the Ficoll-Paque method (Boyum 1968). The DNA was measured using a spectrophotometer and the equivalent of 0.5-2.0μg purified peripheral blood mononuclear cells (PBMNC) DNA were added to the PCR. None of the peripheral blood samples from patients with (n=7) and without (n=10) PML contained detectable JCV DNA, but all contained detectable control pyruvate dehydrogenase human sequences.

5.7. Discussion

This study sought to examine further the association of JCV with PML, and to confirm that methods based on PCR technology can be used to aid virus-specific diagnosis of PML. The detection of JCV sequences in brain tissue was shown in this study to be specific for the disease PML. All the brain tissue from patients with a clinical diagnosis of PML contained JCV DNA, while no JCV sequences could be detected in the frontal lobe samples taken at autopsy from 14 HIV-infected patients without PML. This 100% specificity agreed with the histological findings and therefore can be used for confirmation of PML, although it still relies on the acquisition of brain tissue.

The advantage of looking in the CSF for JCV sequences is that lumbar puncture is a far less invasive procedure than brain biopsy. This study also showed that the detection of JCV in CSF is specific for diagnosis of PML, with 19 (83%) out of 23 of the CSF samples from PML patients showing the presence of JCV DNA. Although this study showed that sensitivity was only 83%, it showed the method to be highly specific because JCV DNA was not amplified from 67 CSF samples from patients without a diagnosis of PML, but with other neurological disorders. (This compares with many other studies and this will be discussed in the general discussion).

Both CSF and brain samples were available from 4 of the PML patients. In 3 of the paired samples, JCV DNA was present in both samples, but in 1 patient JCV was only detected in the autopsy brain sample. The absence of JCV DNA in this CSF sample and others could be due to the sensitivity being dependent on the stage of the disease, whereby at a more advanced stage the increase in JCV replication improves the chance of recovering detectable JCV DNA. The input into the PCR in these studies was 10μl of CSF. Therefore there would be a greater chance of amplifying JCV DNA if a larger volume of CSF could be put into the PCR or a CSF concentration step was included.

This was only tried on 1 sample in this study and in this case there was no recoverable JCV DNA. Also there could be PCR-inhibitory substances in CSF. However, this seems not be the case in this study since JCV DNA was detected in all the negative CSFs when spiked with JCV DNA. Therefore care must be taken in the interpretation of a negative PCR result and, depending on any other available information, it may still be necessary to perform a brain biopsy in order to confirm JCV CNS infection or repeat the lumbar puncture at a later stage.

It was shown in both brain and urine extracts that there was possible inhibition of PCR from the sample. These possible inhibitory factors were easily removed in this study by a dilution stage, although this would lead to a drop in sensitivity in the ability to amplify detectable DNA. An extraction procedure such as the phenol chloroform one used in the extraction of DNA from brain was shown to remove this inhibition, although the amplification stage following this extraction showed a drop in sensitivity. This drop in sensitivity could be due to the DNA extraction efficiency of this particular method. The manufatures (Pharmacia) claim that extraction by Ficoll-Pacque gives a 40% recovery of lymphocytes from normal blood and this may be lower in samples obtained from immunsuppressed patients. In the case of JCV detection in the brain of patients with PML, removal of any inhibitory substances was not considered important because of the high levels obtained in the lesions sampled.

The prevalence of JCV in urine is based on diluting out any possible inhibitory factors. Therefore to increase sensitivity it would be necessary to use an extraction method which removed inhibitory factors. When urine samples containing JCV are diluted down to end point, high levels of amplifiable DNA were shown, and so the numbers would be deemed accurate. Although the low numbers of samples available in these studies can

only give us an indication of the prevalence, but it does show that excretion of JCV in urine is not just limited to patients with PML.

In this study there was no evidence of PCR inhibition by substances in the CSF supernatant. However, if the detection of JCV DNA is to be used diagnostically, sensitivity is important and a method to remove possible inhibitory factors must be considered.

The absence of detectable JCV in PBMNC was not considered to be the result of the extracted sample containing inhibitors of PCR because it was possible to amplify DNA from the pyruvate dehydrogenase human sequences. This finding does not rule out the possibility of JCV DNA being present at undetectable levels or in other fractions of blood.

From these studies it was decided to use PCR as a diagnostic tool in the investigation of PML. When clinical symptoms of PML were observed, a CSF sample is taken to perform a JCV PCR on and the detection of JCV sequences in this sample was believed to be indicative of PML.

Table 5.1. Summary of clinical samples available for this study

Patients	Clinical samples				
	CSF	Brain biopsy	Post-mortem brain tissue	Urine	Peripheral blood
Diagnosed PML HIV +ve n=21 (group1)	21	0	2	8	7
Diagnosed PML HIV -ve n=2 (group1)	2	2	0	0	0
Not diagnosed PML HIV +ve n=67 (group2)	67	0	5	10	10
Not diagnosed PML HIV +ve n=33 (additional controls)	0	0	14	19	0

Table 5.2. JC virus DNA detection in the CSF of patients diagnosed with PML (group 1) and those not diagnosed with PML (group 2)

Patient group	Number of patients	JCV DNA detected	CD4 count range	CD4 count median
Diagnosed PML (Group 1)	23	19	10-165/mm^3	40/mm^3
Not diagnosed PML (Group 2)	67	0	1-200/mm^3	20/mm^3

Table 5.3. Comparison of two DNA extraction methods on post-mortem brain tissue

Patient	Dilution of sample	JCV DNA detected	
		SDS/phenol chloroform	SDS/Boil
GG (diagnosed PML)	Neat	+	-
	1×10^{-1}	+	+
	1×10^{-2}	+	+
	1×10^{-3}	+	+
	1×10^{-4}	+	+
	1×10^{-5}	+	+
	1×10^{-6}	+	+
	1×10^{-7}	-	+
	1×10^{-8}	-	-
MT (not diagnosed PML)	Neat	-	-
	1×10^{-1}	-	-
	1×10^{-2}	-	-
	1×10^{-3}	-	-
	1×10^{-4}	-	-
RC (not diagnosed PML)	Neat	-	-
	1×10^{-1}	-	-
	1×10^{-2}	-	-
	1×10^{-3}	-	-
	1×10^{-4}	-	-

Table 5.4. JCV DNA detection in ante-mortem and post-mortem samples from patients diagnosed with PML

Patient	HIV-infected	JCV DNA detected in anti-mortem CSF		JCV DNA detected in brain (type of sample)
		supernatant	pellet	
SJ	-	-	+	+ (ante-mortem brain biopsy)
JB	-	+	+	+ (ante-mortem brain biopsy)
GG	+	-	+	+ (autopsy brain sample)
PK	+	-	-	+ (autopsy brain sample)

Table 5.5. Detection of JCV DNA in urine and peripheral blood mononuclear cells

Patient Group (group, patient numbers)	JCV DNA detected in clinical samples	
	Urine	Peripheral blood mononuclear cells
Diagnosed PML (group 1, n=8)	3/8	0/7 (no sample available from one patient)
Not diagnosed PML (group 2, n=10)	1/10	0/10
Additional control samples (n=19)	2/19	no samples available

Chapter 6: Sequence analysis of the JCV genome

6.1. Introduction

Although a considerable diversity in the regulatory region sequence has been reported among JCV isolates from the brain of PML patients, the rest of the genome is remarkably conserved (Loeber *et al* 1988; Yogo *et al* 1991; Ault and Stoner 1994). It has been suggested that JCV undergoes genome alterations consisting of deletions and duplications when it is adapting to growth in brain tissue (Yogo *et al* 1993). It is generally believed that this rearranged type is responsible for PML, although it is unclear whether it is due to reactivation of latent virus or primary infection. In some studies this rearranged type has been found only in the brain, while others have shown it to be present also in the kidney and urine. To help answer these questions genome sequence studies were performed on brain and urine samples taken from patients with and without PML. The aim was to amplify the regulatory region of JCV DNA in each sample, determine the nucleotide sequence by manual dideoxy sequencing and compare the JCV regulatory region found at different sites in the body by alignment of these sequences. The final aim was to compare the sequences with the archetype and prototype form of JCV.

6.2. Patients and samples

Six JCV DNA sequences (A1 to A6) were rescued from the brain (3) and urine (3) of four immunosuppressed patients (Table 6.1). Patient JB was HIV-uninfected and immunosuppressed because of undergoing therapy for haematological malignancies.

This patient had been diagnosed with PML on the basis of magnetic resonance imaging (MRI) and clinical investigations. An ante-mortem brain biopsy was performed to confirm the diagnosis. Three patients were immunosuppressed because of HIV disease. Two of these, GG and PK had been diagnosed with PML on the basis of MRI, clinical symptoms and JCV DNA detection in their cerebrospinal fluid (CSF). These ante-mortem CSF samples and additional urine samples were taken approximately 5 (GG) and 11 (PK) weeks before the post-mortem. Patients GG and PK were confirmed to have PML at the post-mortem and during this examination frontal lobe brain tissue was taken. The third HIV-infected patient MC was part of the control group from the London PML study (group 2 described in section 5.2), which comprised immunosuppressed patients who presented with acute neurological symptoms and signs but who were not deemed to have PML. Urine samples were available from 10 patients in this group and all were screened for the presence of JCV DNA. This was only detected in the urine of patient MC.

6.3. Design of amplification and sequencing primers

Primers were designed to amplify by nested PCR a region of the JCV genome which included the 393 base pair regulatory region (Table 6.2). The oligonuceotide sequences of first round primers P1 and P2 were selected to amplify a 905 base pair product. Then primers P3 and P4 were used to amplify a nested PCR product of 683 base pairs long (Fig. 6.1). The primer P3 was biotinylated so that the PCR product could be captured onto a streptavidin coated solid phase. Three sequencing primers (S1-3) were designed to complement regions on the nested PCR product. They were sited at specific points on the genome at 160-173 base pair intervals and in areas where no deletions would be expected. Therefore by sequencing individually with each primer one would expect to

read a sequence of over 500 base pairs starting at 100 base pairs downstream from the regulatory region. The PCR and the sequencing primers were screened by FASTA (Pearson and Lipman, 1988) on the world wide web accessed from the European Bioinformatics Institute (EBI) home page.

6.4. Preparation and amplification of JCV DNA from brain tissue and urine

The DNA was extracted from brain tissue using the sodium dodecyl sulphate (SDS) lysis method and the lysed cell preparations were boiled. Tenfold dilutions were made of the extracted DNA and 1μl put directly into the first round PCR mix to make a total volume of 50μl. All PCRs had final concentrations of 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris (pH 8.3), 200 μM dNTP, 1.25 unit of AmpliTaq DNA polymerase (Perkin Elmer) and 100 ng of each primer. For the first round the initial denaturation at 94^{o}C for 4 minutes followed by 35 cycles of 94^{o}C for 1 minute (denaturation), 52^{o}C for 1 minute (annealing), and 72^{o}C for 1 minute (extension), with an additional 7 minute extension. After completion of the 35 cycles 1μl of the first round product was transferred to the second round PCR mixture with a total volume of 100μl. The second amplification was 30 cycles of 94^{o}C for 1 minute (denaturation), 48^{o}C for 1 minute (annealing), and 72^{o}C for 1 minute (extension), with an additional 7 minute extension. Ten microlitres of the second round product was visualised by electrophoresis on a 2% ethidium bromide stained agarose gel. Where it was necessary to perform a third round PCR this was carried out by transferring 1μl of the second round amplification into a fresh PCR reaction mixture (identical to the second round mixture) and repeating the second round amplification.

The DNA was extracted from the urine by the heat extraction method outlined in section 2. Tenfold dilutions were made of the extracted DNA and 10μl of each dilution put directly into the first round PCR mix to make a total volume of 50μl. Amplification was performed using the same conditions that were used for the brain extract.

The biotinylated PCR products were purified by capturing 70μl of the PCR product directly onto streptavidin coated magnetic particles. This method is described in the general methods.

6.5. Sequencing of single stranded biotinylated PCR products

Manual dideoxy sequencing was carried out on each biotinylated PCR product with the three sequencing primers S1-S3. The general method is described in chapter 2. In order to read the sequence close to primer S3, the PCR product was also sequenced using the 'inner primer' P4 as the sequencing primer. All the sequencing was carried out more than once. The resulting sequences obtained from each sample were manually aligned so that each section overlapped the next section and the resulting sequence was manually typed into Microsoft Word documents.

6.6. Published sequences

For comparison two additional sequences were down loaded from the world wide web. The first sequence A7 was the regulatory region of CY, the archetype form of JCV (D00801). CY was originally found in the urine of a Japanese patient and is generally believed to be distinct from those often isolated from PML brains (Yogo *et al* 1990). The second was the regulatory region of Mad1 the prototype JCV isolate (J02226), described by Frisque and colleagues in 1984, isolated from the brain of a patient with PML. The basic difference between the regulatory region sequence of these two types is

that the prototype has deletions of 23 and 64-66 nucleotides and a sequence of 98 base pairs is duplicated.

6.7. Results

6.7.1. Comparison of sequence lengths obtained from six JCV amplicons

After amplification by the nested PCR, the length of the DNA fragments was determined by electrophoresis though ethidium bromide stained NuSieve agarose (FMC, Rockland, USA). The bands on the gel were visualised every 30 minutes up to 2.5 hours and compared with a DNA marker. After one hour it was possible to determine that five of the DNA fragments (A1, A2, A3, A4 and A6) were approximately the expected 683 base pairs long, with the fragment A5 being smaller. After 2.5 hours it was possible to see that A5 was approximately 40 base pairs smaller with only slight differences between the other fragments. After lining up the sequences obtained using the primers S1-3 it was also possible to compare the size by the number of base pairs in the regulatory region. The sequences were edited so there were three base pairs on each side of the regulatory region. The resulting lengths of the regulatory region for each sequence were 396 base airs (A1), 384 base pairs (A2), 384 base pairs (A3), 387 base pairs (A4), 340 base pairs (A5) and 384 base pairs (A6).

6.7.2. Alignment and comparison of the six regulatory region sequences obtained from the JCV amplicons (A1 to A6)

The regulatory sequences obtained from samples A1-A6 were multiply aligned along with A7 and A8 using ClustalW (Thompson *et al* 1994) accessed from the EBI home page (Fig. 6.2). The isolates obtained from the urine and brain of HIV infected patients with PML (A1-A4) and the isolate from the urine of a HIV infected patient without PML

(A6) all had similar regulatory sequences. These were consistently different from that seen in the Mad1 isolate (A8) and the JCV isolate obtained from the brain of a patient (with PML) after a bone marrow transplant (A5).

To help clarify the differences between these isolates the sequences of A1 and A5 were separately aligned against the Mad1 prototype (A8) and displayed schematically in Fig. 6.3. The regulatory regions of these three were clearly different due to deletions and insertions. The sequences from A1 and A5 had a 23 base pair insertion TAGGGAGGAGCTGGCTAAAACTG which was not present in the Mad1 prototype. There was a 98 base pair repeat insertion present in the Mad1, which was not present in isolates obtained from patients A1 and A5. However, the sequences obtained at this point in the genome also differed between A1 (PML +ve, HIV +ve) and A5 (PML +ve, HIV -ve). The 98 base pair repeat insertion meant that the regularity region of Mad1 contained two TATA boxes.

The regulatory region of CY was multiply aligned with A1 and A5 (Fig. 6.4). The archetype JCV (A7) sequence was almost identical with the isolate taken in this study from the brain of an HIV infected patient with PML (A1). The main difference was that A1 had a 13 base pair repeat insert TAGTCCTTAATCA which was also found in two other sequences obtained in this study from HIV infected patients. In comparison with the archetype CY the sequence obtained from the bone marrow transplant patient (A5) had a 70 base pair deletion replaced with a 25 base pair insertion (Fig. 6.4).

For comparison the sequences of different isolates found in the urine and brain of the HIV infected patients were multiply aligned (Fig. 6.5). This included paired brain and urine sequences from two patients (A1/2 and A3/4) and urine sequence from the control patient (A6). The regulatory region sequence isolated from the post-mortem brain of the patient GG contained the additional 13 base pair insertion TAGTCCTTAATCA which

was not present in the sequence amplified from an ante-mortem urine sample taken from the same patient 5 weeks earlier. Both the brain and the urine isolate from patient PK contained this insertion (A3 and A4), but in A3 there was a possible three base pair deletion. A 9 base pair deletion GTTTTGGCT occurred in the sequences obtained from both the urine and brain of patient PK, but this was not seen in any of the other sequences. A homology search on the sequences A3 and A4 looking for JCV regulatory sequences with close homology showed no others to have this deletion.

6.8. Discussion

The JC virus has been divided into various variants depending on the rearrangement of the regulatory region. These are generally based around the prototype (Mad1) and the archetype (CY) form of JCV. In this study, the structural organisation of the regulatory sequence in the five isolates from PML patients were distinct from the prototype Mad1. Since three of these sequences were determined from JCV DNA extracted and amplified direct from the brain of PML patients, it suggests that other variants can be present apart from the prototype. It has been reported that there can be a mixture of variants at one site in the body, especially in the brain of a PML patient (Yogo *et al* 1994). In this study only one JCV sequence was determined for each sample although, because it was from an end point dilution, it was presumed to be from the variant in the higher copy numbers. Therefore even though only one sequence was determined from each sample, it does not rule out the possibility of a heterogeneous JCV population.

There were a few single nucleotide substitutions between the sequences and it is necessary to be careful in the interpretation of these because the nucleotide sequence data is derived from templates produced by PCR. The DNA polymerase (Ampli Taq) used can have an error rate that would be increased by the multiple amplification

procedure of PCR (Smith *et al* 1997). Therefore in this study it was decided to concentrate on the interpretation of the main deletions and insertions of the nucleotide sequences.

Four of the isolates from PML patients had a similar sequence to the archetype CY (A1-4), but three of them showed small deletions or insertions. Isolate A2 (from urine) showed the closest similarity with the archetype CY and this regulatory sequence has been reported many times in isolates obtained from urine and occasionally in brain (Yogo *et al* 1994). The same sequence (A6) was also isolated in the urine of a patient without PML suggesting that this variant can be present in patients without clinical evidence of PML, although this patient was HIV-infected and immunosuppresed. The two sequences (A1 & A6) which bear the closest similarity with CY were only found in isolates detected in urine.

Isolates A1 (brain), A3 (brain) and A4 (urine) contain a 13 base pair sequence repeat, which has been reported only once before in the urine of a healthy patient (D00806, unpublished data submitted to a database by Yogo). Interestingly, the sequence A1 and A2 came respectively from the brain and urine of patient GG and this 13 base pair repeat sequence is only seen in the brain isolate, although the urine sample was taken five weeks before and therefore there is a possibility of genomic rearrangement in this time. Sequences A3 and A4 come respectively from the brain and urine of patient PK and have almost identical regulatory sequences, the only difference being a 3 base pair deletion in A3. This 3 base pair deletion is part of the 13 base insertion and it is not clear if this is part of genomic rearrangement or a possible error in the sequencing. PK contains a further 9 base pair deletion present in the sequences obtained from both urine and brain. This deletion appears to be specific to the DNA rescued from this patient and no other studies have shown this.

All the sequences discussed so far were from JCV detected in HIV-infected patients and they clearly had a closer homology to the regulatory sequences archetype CY than the prototype Mad1. None of them had the 23 or 64-66 base pair deletions associated with Mad1. The sequence A5 was also distinct from the prototype Mad1 in that it did not have the 23 base pair deletion, but it is also distinct from the archetype CY in that it had a deletion of 70 base pairs with a 25 base pair insertion. This DNA sequence was rescued from the brain of patient JB who been diagnosed with PML after undergoing a bone marrow transplantation. The deletions and insertions could represent the initial stage of adaptation from one form of JCV to another or represent another variant. This particular regulatory sequence has not been reported before.

In conclusion it appears in this study that the JCV regulatory sequences rescued from patients infected with HIV have a closer homology to the archetype CY. This is in contrast to most other studies. However the possibility of heterogeneous populations cannot be ruled out. One other factor to be considered is that most other sequencing studies have been carried out after cloning, while in this study the direct sequencing of PCR products allows the generation of DNA sequence data directly from the patient.

Table 6.1. Patients and tissue containing JC virus

Sample number	Patient	Type of Sample	HIV status	Diagnosed with PML
A1	GG	post-mortem brain	+ve	yes
A2	GG	ante-mortem urine*	+ve	yes
A3	PK	post-mortem brain	+ve	yes
A4	PK	ante-mortem urine**	+ve	yes
A5	JB	ante-mortem brain biopsy	-ve	yes
A6	MC	urine	+ve	no

* this sample was taken 5 weeks before the post-mortem.

** this sample was taken 11 weeks before the post-mortem.

Table 6.2. PCR oligonucleotide primers for amplification and sequencing of the JCV regulatory region

Oligonucleotide	Sequence (5'→3')	Nucleotide position
P1	TACCAAAATCAGGCTGAGC	4788-4809
P2	GCAACTAGGTCCCCCAAAAG	563-544
P3	GACAGGAATGTTCCCCCATG	4928-4946
P4	GGTTCAGGCAAAGCACTGTAT	479-459
S1	AAAAGTCCAGCAAAAATTCT	394-375
S2	CATGTTCCCTTGGCTGCTTT	235-216
S3	CATGCTTGGCTGGCAGCCAT	38-57

Fig. 6.1. Schematic representation of the location of the sequencing primers on the JCV genome

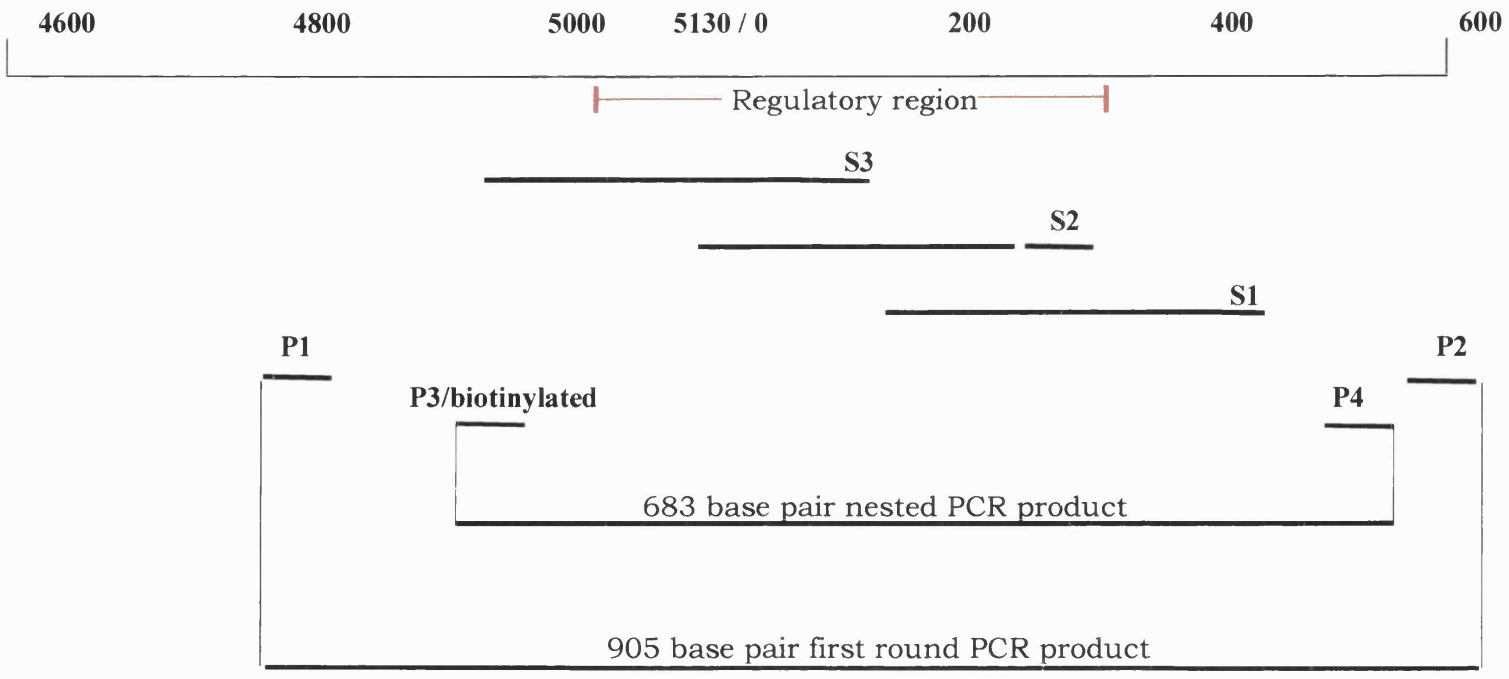


Fig. 6.2. Alignment of genome sequences from the regulatory region of the JC virus

```
A3      CATTTTAGCTTTTTGCAGCAAAAAATTACTGCAAAAAAGGGAAAAACAAGGGAATTTCCC 60
A4      CATTTTAGCTTTTTGCAGCAAAAAATTACTGCAAAAAAGGGAAAAACAAGGGAATTTCCC 60
A1      CATTTTAGCTTTTTGCAGCAAAAAATTACTGCAAAAAAGGGAAAAACAAGGGAATTTCCC 60
A2      CATTTTAGCTTTTTGCAGCAAAAAATTACTGCAAAAAAGGGAAAAACAAGGGAATTTCCC 60
A6      CATTTTAGCTTTTTGCAGCAAAAAATTACTGCAAAAAAGGGAAAAACAAGGGAATTTCCC 60
A7      CATTTTTGCTTTTTGTAGCAAAAAATTAGTGCAAAAAAGGGAAAAACAAGGGAATTTCCC 60
A5      CATTTTAGCTTTTTGCAGCAAAAAATTACTGCAAAAAAGGGAAAAACAAGGGAATTTCCC 60
A8      CATTTTAGCTTTTTGCAGCAAAAAATTACTGCAAAAAAGGGAAAAACAAGGGAATTTCCC 60
        ****** ******** ************ ******************************

A3      TGGCCTCCTAAAAAGCCTCCACGCCCTTACTACTTCTGAGTAAGCTTGGAGGCGGAGGCG 120
A4      TGGCCTCCTAAAAAGCCTCCACGCCCTTACTACTTCTGAGTAAGCTTGGAGGCGGAGGCG 120
A1      TGGCCTCCTAAAAAGCCTCCACGCCCTTACTACTTCTGAGTAAGCTTGGAGGCGGAGGCG 120
A2      TGGCCTCCTAAAAAGCCTCCACGCCCTTACTACTTCTGAGTAAGCTTGGAGGCGGAGGCG 120
A6      TGGCCTCCTAAAAAGCCTCCACGCCCTTACTACTTCTGAGTAAGCTTGGAGGCGGAGGCG 120
A7      TGGCCTCCTAAAAAGCCTCCACGCCCTTACTACTTCTGAGTAAGCTTGGAGGCGGAGGCG 120
A5      TGGCCTCCTAAAAAGCCTCCACGCCCTTACTACTTCTGAGTAAGCTTGGAGGCGGAGGCG 120
A8      TGGCCTCCTAAAAAGCCTCCACGCCCTTACTACTTCTGAGTAAGCTTGGAGGCGGAGGCG 120
        ************************************************************

A3      GCCTCGGCCTCCTGTATATATAAAAAAAAGGGAAGGTAGGGAGGAGCTGGCTAAAACTGG 180
A4      GCCTCGGCCTCCTGTATATATAAAAAAAAGGGAAGGTAGGGAGGAGCTGGCTAAAACTGG 180
A1      GCCTCGGCCTCCTGTATATATAAAAAAAAGGGAAGGTAGGGAGGAGTAGGCTAAAACTGG 180
A2      GCCTCGGCCTCCTGTATATATAAAAAAAAGGGAAGGTAGGGAGGAGCTGGCTAAAACTGG 180
A6      GCCTCGGCCTCCTGTATATATAAAAAAAAGGGAAGGTAGGGAGGAGCTGGCTAAAACTGG 180
A7      GCCTCGGCCTCCTGTATATATAAAAAAAAGGGAAGGTAGGGAGGAGCTGGCTAAAACTGG 180
A5      GCCTCGGCCTCCTGTATATATAAAAAAAAGGGAAGGTAGGGAGGAGCTGGCTAAAACTGG 180
A8      GCCTCGGCCTCCTGTATATATAAAAAAAAGGGAA--------GG---------------G 157
        ********************************        **               *

A3      ATGGCTGCCAGCCAAGCATGAGCTCATACCTAGGGAGCCAACCAGCTGACAGCCAG---A 237
A4      ATGGCTGCCAGCCAAGCATGAGCTCATACCTAGGGAGCCAACCAGCTGACAGCCAG---A 237
A1      ATGGCTGCCAGCCAAGCATGAGCTCATACCTAGGGAGCCAACCAGCTGACAGCCAG---A 237
A2      ATGGCTGCCAGCCAAGCATGAGCTCATACCTAGGGAGCCAACCAGCTGACAGCCAG---A 237
A6      ATGGCTGCCAGCCAAGCATGAGCTCATACCTAGGGAGCCAACCAGCTGACAGCCAG---A 237
A7      ATGGCTGCCAGCCAAGCATGAGCTCATACCTAGGGAGCCAACCAGCTGACAGCCAG---A 237
A5      ATGGCTGCCAGCCAAGCATGAGCTCATACCTAGGGAGCCAACCAGCTGACT--------- 231
A8      ATGGCTGCCAGCCAAGCATGAGCTCATACCTAGGGAGCCAACCAGCTAACAGCCAGTAAA 217
        ********************************************** **

A3      GGGAGC-CCTGGCTGCATGC-------------------CACTGGCAGTTATAGTG-AA 275
A4      GGGAGC-CCTGGCTGCATGC-------------------CACTGGCAGTTATAGTG-AA 275
A1      GGGAGC-CCTGGCTGCATGC-------------------CACTGGCAGTTATAGTG-AA 275
A2      GGGAGC-CCTGGCTGCATGC-------------------CACTGGCAGTTATAGTG-AA 275
A6      GGGAGC-CCTGGCTGCATGC-------------------CACTGGCAGTTATAGTG-AA 275
A7      GGGAGC-CCTGGCTGCATGC-------------------CACTGGCAGTTATAGTG-AA 275
A5      -----C-CTTAATCACA----------------------CA----------AGTA-AA 250
A8      CAAAGCACAAGGCTGTATATATAAAAAAAAGGGAAGGGATGGCTGCCAGCCA-AGCATGA 276
             * *        *                                  **    *
```

Fig. 6.2. ***continued***

```
A3      ACCCCTCCCATAGTCCTTAATCATAGTC--CT---T---CACAAGTAAACAAAGCACAAG 327
A4      ACCCCTCCCATAGTCCTTAATCATAGTC--CT---TAATCACAAGTAAACAAAGCACAAG 330
A1      ACCCCTCCCATAGTCCTTAATCATAGTC--CT---TAATCACAAGTAAACAAAGCACAAG 330
A2      ACCCCTCCC-----------CATAGTC--CT---TAATCACAAGTAAACAAAGCACAAG 318
A6      ACCCCTCCC-----------CATAGTC--CT---TAATCACAAGTAAACAAAGCACAAG 318
A7      ACCCCTCCC------------ATAGTC--CT---TAATCACAAGTAAACAAAGCACAAG 317
A5      --------------------CAAAG----C-----------AAGTAAACAAAGCACAAG 274
A8      GCTCATACC-TAG---------GGAGCCAACCAGCTAACAGCCAGTAAACAAAGCACAAG 326
                              **    *             ****************


A3      GGGAAGTGGAAAGCAGCCAAGGGAACATGTTT-GCGAGCCAGAGCT---------TGTCA 377
A4      GGGAAGTGGAAAGCAGCCAAGGGAACATGTTT-GCGAGCCAGAGCT---------TGTCA 380
A1      GGGAAGTGGAAAGCAGCCAAGGGAACATGTTT-GCGAGCCAGAGCTGTTTTGGCTTGTCA 389
A2      GGGAAGTGGAAAGCAGCCAAGGGAACATGTTT-GCGAGCCAGAGCTGTTTTGGCTTGTCA 377
A6      GGGAAGTGGAAAGCAGCCAAGGGAACATGTTT-GCGAGCCAGAGCTGTTTTGGCTTGTCA 377
A7      GGGAAGTGGAAAGCAGCCAGGGGAACATGTTTTGCGAGCCAGAGCTGTTTTGGCTTGTCA 377
A5      GGGAAGTGGAAAGCAGCCAAGGGAACATGTTT-GCGAGCCAGAGCTGTTTTGGCTTGTCA 333
A8      GGGAAGTGGAAAGCAGCCAAGGGAACATGTTTTGCGAGCCAGAGCTGTTTTGGCTTGTCA 386
        ******************* ************ *************          *****


A3      CCAGCTGGCCATG 390
A4      CCAGCTGGCCATG 393
A1      CCAGCTGGCCATG 402
A2      CCAGCTGGCCATG 390
A6      CCAGCTGGCCATG 390
A7      CCAGCTGGCCATG 390
A5      CCAGCTGGCCATG 346
A8      CCAGCTGGCCATG 399
        *************
```

Sample A3 Isolated from the brain of a HIV-infected patient diagnosed with PML
Sample A4 Isolated from the urine of a HIV-infected patient diagnosed with PML
Sample A1 Isolated from the brain of a HIV-infected patient diagnosed with PML
Sample A2 Isolated from the urine of a HIV-infected patient diagnosed with PML
Sample A6 Isolated from the urine of a HIV-infected patient
Sample A7 CY, the archetype form of JCV (Yogo *et al*, 1990)
Sample A5 Isolated from the brain of a patient post bone marrow transplant diagnosed with PML
Sample A8 Mad1, the prototype JCV isolate (J02226, Frisque *et al* 1984)

The sequences are aligned 5'→3' and the numbering includes three base pairs at each end of the regulatory region.

*Indicates alignment between all 8 sequences

Fig. 6.3. Sequences of the JCV regulatory region from isolated from three brains of patients with PML

```
A8      CATTTTAGCTTTTTGCAGCAAAAAATTACTGCAAAAAAGGGAAAAACAAGGGAATTTCCCTGGCCTCCTAAAAAGCCTCCACGCCCTTAC
A1      CATTTTAGCTTTTTGCAGCAAAAAATTACTGCAAAAAAGGGAAAAACAAGGGAATTTCCCTGGCCTCCTAAAAAGCCTCCACGCCCTTAC
A5      CATTTTAGCTTTTTGCAGCAAAAAATTACTGCAAAAAAGGGAAAAACAAGGGAATTTCCCTGGCCTCCTAAAAAGCCTCCACGCCCTTAC

A8      TACTTCTGAGTAAGCTTGGAGGCGGAGGCGGCCTCGGCCTCCTGTATATATAAAAAAAAGGGAAGG----------------------G
A1      TACTTCTGAGTAAGCTTGGAGGCGGAGGCGGCCTCGGCCTCCTGTATATATAAAAAAAAGGGAAGGTAGGGAGGAGCTGGCTAAAACTGG
A5      TACTTCTGAGTAAGCTTGGAGGCGGAGGCGGCCTCGGCCTCCTGTATATATAAAAAAAAGGGAAGGTAGGGAGGAGCTGGCTAAAACTGG

A8      ATGGCTGCCAGCCAAGCATGAGCTCATACCTAGGGAGCCAACCAGCTAACAGCCAGTAAACAAAGCACAAGGCTGTATATATAAAAAAAA
A1      ATGGCTGCCAGCCAAGCATGAGCTCATACCTAGGGAGCCAACCAGCTGACAGCCAG------------------AGGGAGCCCTGGCTAG
A5      ATGGCTGCCAGCCAAGCATGAGCTCATACCTAGGGAGCCAACCAGCT----------------------------------------

A8      GGGAAGGGATGGCTGCCAGCCAAGCATGAGCTCATACCTAGGGAGCCAACCAGCTAACAGCCAGTAAACAAAGCACAAGGGGAAGTGGAA
A1      CATGCCACTGGCAGTTATAGTGAAACCCCTCCCATAGTCCTTAATCATAGTCCTTAATCACAAGTAAACAAAGCACAAGGGGAAGTGGAA
A5      ------------------------------GACTCCTTAATCACACAAGTAAACAAAGCAAGTAAACAAAGCACAAGGGGAAGTGGAA

A8      AGCAGCCAAGGGAACATGTTTGCGAGCCAGAGCTGTTTTGGCTTGTCACCAGCTGGCCATG 399
A1      AGCAGCCAAGGGAACATGTTTGCGAGCCAGAGCTGTTTTGGCTTGTCACCAGCTGGCCATG 402
A5      AGCAGCCAAGGGAACATGTTTGCGAGCCAGAGCTGTTTTGGCTTGTCACCAGCTGGCCATG 346
```

Sample A8 Mad1, the prototype JCV isolate (J02226, Frisque *et al* 1984).
Sample A1 Isolated from the brain of a HIV-infected patient diagnosed with PML.
Sample A5 Isolated from the brain of a patient post bone marrow transplant diagnosed with PML.
Nucleotides highlighted red or green are those that differ from the sequence of A8.
The underlined nucleotides represent the 98 base pair repeat in Mad1.

Fig. 6.4. Comparison of two JCV regulatory region sequences isolated from the brain of PML patients, with the archetype form of JCV

```
A7      CATTTTTGCTTTTTGTAGCAAAAAATTAGTGCAAAAAAGGGAAAAACAAGGGAATTTCCCTGGCCTCCTAAAAAGCCTCCACGCCCTTAC
A1      CATTTTAGCTTTTTGCAGCAAAAAATTACTGCAAAAAAGGGAAAAACAAGGGAATTTCCCTGGCCTCCTAAAAAGCCTCCACGCCCTTAC
A5      CATTTTAGCTTTTTGCAGCAAAAAATTACTGCAAAAAAGGGAAAAACAAGGGAATTTCCCTGGCCTCCTAAAAAGCCTCCACGCCCTTAC

A7      TACTTCTGAGTAAGCTTGGAGGCGGAGGCGGCCTCGGCCTCCTGTATATATAAAAAAAAGGGAAGGTAGGGAGGAGCTGGCTAAAACTGG
A1      TACTTCTGAGTAAGCTTGGAGGCGGAGGCGGCCTCGGCCTCCTGTATATATAAAAAAAAGGGAAGGTAGGGAGGAGTAGGCTAAAACTGG
A5      TACTTCTGAGTAAGCTTGGAGGCGGAGGCGGCCTCGGCCTCCTGTATATATAAAAAAAAGGGAAGGTAGGGAGGAGCTGGCTAAAACTGG

A7      ATGGCTGCCAGCCAAGCATGAGCTCATACCTAGGGAGCCAACCAGCTGACAGCCAGAGGGAGCCCTGGCTGCATGCCACTGGCAGTTATA
A1      ATGGCTGCCAGCCAAGCATGAGCTCATACCTAGGGAGCCAACCAGCTGACAGCCAGAGGGAGCCCTGGCTGCATGCCACTGGCAGTTATA
A5      ATGGCTGCCAGCCAAGCATGAGCTCATACCTAGGGAGCCAACCAGCTGACTCCTTAATCACACAAGTAAACAAAG---------------

A7      GTGAAACCCCTCCCATAGTCCTTAATCA-------------CAAGTAAACAAAGCACAAGGGGAAGTGGAAAGCAGCCAGGGGAACATGT
A1      GTGAAACCCCTCCCATAGTCCTTAATCATAGTCCTTAATCACAAGTAAACAAAGCACAAGGGGAAGTGGAAAGCAGCCAAGGGAACATGT
A5      ----------------------------------------CAAGTAAACAAAGCACAAGGGGAAGTGGAAAGCAGCCAAGGGAACATGT

A7      TTTGCGAGCCAGAGCTGTTTTGGCTTGTCACCAGCTGGCCATG 390
A1      TT-GCGAGCCAGAGCTGTTTTGGCTTGTCACCAGCTGGCCATG 402
A5      TT-GCGAGCCAGAGCTGTTTTGGCTTGTCACCAGCTGGCCATG 346
```

Sample A7 CY, the archetype form of JCV (Yogo *et al*, 1990)
Sample A1 Isolated from the brain of a HIV-infected patient diagnosed with PML
Sample A5 Isolated from the brain of a patient post bone marrow transplant diagnosed with PML
Nucleotides highlighted red or green are those that differ from the sequence of CY.
The underlined nucleotides represent the 13 base pair repeat in A1.

Fig. 6.5. Comparison between a section of the JCV regulatory region sequences isolated from the brain and urine of patients infected with HIV and the archetype form of JCV

```
A7      AAACCCCTCC-CATAGTCCTTAATCA-------------CAAGTAAACAAAGCACAAGGGGAAGTG
A3      AAACCCCTCC-CATAGTCCTTAATCATAGTCCTT---CACAAGTAAACAAAGCACAAGGGGAAGTG
A4      AAACCCCTCC-CATAGTCCTTAATCATAGTCCTTAATCACAAGTAAACAAAGCACAAGGGGAAGTG
A1      AAACCCCTCC-CATAGTCCTTAATCATAGTCCTTAATCACAAGTAAACAAAGCACAAGGGGAAGTG
A2      AAACCCCTCCCCATAGTCCTTAATCA-------------CAAGTAAACAAAGCACAAGGGGAAGTG
A6      AAACCCCTCCCCATAGTCCTTAATCA-------------CAAGTAAACAAAGCACAAGGGGAAGTG

A7      GAAAGCAGCCAGGGGAACATGTTTTGCGAGCCAGAGCTGTTTTGGCTTGTCACCAGCTGGCCATG
A3      GAAAGCAGCCAAGGGAACATGT-TTGCGAGCCAGAGCT---------TGTCACCAGCTGGCCATG
A4      GAAAGCAGCCAAGGGAACATGT-TTGCGAGCCAGAGCT---------TGTCACCAGCTGGCCATG
A1      GAAAGCAGCCAAGGGAACATGT-TTGCGAGCCAGAGCTGTTTTGGCTTGTCACCAGCTGGCCATG
A2      GAAAGCAGCCAAGGGAACATGT-TTGCGAGCCAGAGCTGTTTTGGCTTGTCACCAGCTGGCCATG
A6      GAAAGCAGCCAAGGGAACATGT-TTGCGAGCCAGAGCTGTTTTGGCTTGTCACCAGCTGGCCATG
```

Sample A7 CY, the archetype form of JCV (Yogo *et al*, 1990)
Sample A3 Isolated from the brain of PK a HIV-infected patient diagnosed with PML
Sample A4 Isolated from the urine of PK a HIV-infected patient diagnosed with PML
Sample A1 Isolated from the brain of GG a HIV-infected patient diagnosed with PML
Sample A2 Isolated from the urine of GG a HIV-infected patient diagnosed with PML
Sample A6 Isolated from the urine of MC a HIV-infected patient
The underlined nucleotides represent the 13 base pair repeated
Nucleotides highlighted red represent deletions/insertions

Chapter 7: Detection and genotyping of human papillomavirus DNA in cervical cells taken from women attending a colposcopy clinic

7.1. Introduction

Many methods have been developed for HPV detection and these vary widely in sensitivity and specificity. DNA amplification by PCR has shown to be a sensitive method of HPV detection (Kuypers *et al* 1993; Morris *et al* 1990; Ward *et al* 1990). There are individual PCR assays for HPV genotype determination which are highly specific. The problem with these separate amplifications is, because of the large number of HPV genotypes, it is time consuming. Therefore, there is great advantage in a single assay being able to amplify all the HPV genotypes with consensus primers. A number of methods have been described using consensus primers to amplify HPV DNA (Manos *et al* 1989; Snijders *et al* 1990; Kleter *et al* 1998).

For genotyping after amplification various methods have been developed either to define the individual genotype or differentiate between high and low risk types. These include restriction fragment length polymorphism (Bernard *et al* 1994), detection by a cocktail of probes specific for high risk genotypes (Jacobs *et al* 1997) and methods depending on detection by specific probes immobilised onto strips (Gravitt *et al* 1998; Kleter *et al* 1999).

The aim of this section is to look at detection of HPV DNA by PCR. Two consensus primer sets were used and any amplified HPV DNA from either PCR was genotyped using a reverse hybridisation line probe assay (LiPA). HPV are found at various sites in the human body. The study described in this section looks at those found in the

genital region and principally those in the high risk group that are being implicated in the development of cervical carcinoma. Therefore the samples were collected consecutively from women who had been referred to the colposcopy clinic at UCL Hospital.

7.2. Patients and samples

Cervical cells were available from 100 women attending the colposcopy clinic of UCL hospital. The women were attending this clinic for one of three reasons:

1) Women attending the colposcopy clinic for the first time referred from the national screening programme according to NHSCSP guidelines (Duncan 1997).

2) Women attending the colposcopy clinic for treatment of cervical dysplasia.

3) Women attending the colposcopy clinic after treatment for cervical dysplasia.

At the time of the colposcopy a smear was taken with an Ayre spatula and cervical cells were placed onto a slide that was sent for routine cytological analysis. The end of the spatula was then broken off and placed in a universal container with 2ml of normal saline. The cervical cells were removed by agitation. The spatula was removed and the suspension spun at 2000g for 5 minutes. The pelleted cells were resuspended in 2ml saline and the centrifugation step was repeated. The cells were resuspended in 200µl saline and the DNA extracted using the Qiagen spin column method described in chapter 2. The extracted DNA was resuspended in 200µl DNAase-free water (Promega, Southampton, UK) and stored at -70°C. The samples were thawed immediately before testing for the presence of HPV DNA and 10µl were

used in each type of PCR with a 50µl final reaction volume. All PCRs were performed on the same thermal cycler.

7.3. Comparison of two PCR primer sets used to detect HPV DNA

Two different primer sets with separate PCR reactions were used in this study.

7.3.1. Manos primer set

The first primer set consisted of MY09 and MY11 which are degenerate primers designed to amplify a 450 base pair fragment located in the L1 region of the HPV genome (Table 7.1). These primers were designed by Manos and colleagues (1989) and are often referred to as the Manos primers. The primers were synthesised by Oswel (Southampton, UK) and, in order for the amplified PCR product to be typed, it was necessary to have a biotin molecule attached at the 5' end of the degenerate MY09 primer. This PCR had final concentrations of 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris (pH 8.3), 200 μM dNTP, 1.25 unit of AmpliTaq Gold DNA polymerase (Perkin Elmer) and 100 ng of each primer. Initial denaturation was at 95°C for 12 minutes followed by 38 cycles of 95°C for 1 minute (denaturation), 55°C for 1 minute (annealing), and 72°C for 1 minute (extension), with an additional 7 minute extension. The products were electrophoresed through a 3% agarose gel, stained with ethidium bromide and visualised under ultraviolet light. The sensitivity of this PCR was accessed with a full length HPV16 sequence cloned in the plasmid pBR 322 (provided by E-M deVilliers, Heidelberg, Germany). Using a dilution series of this plasmid it was shown that this PCR could detect down to 100 HPV copies/ml.

7.3.2. SPF10 primer set

The second primer set SPF10 contained a mixture of 10 sequences (Table 7.1) designed to amplify a 65 base pair fragment (Kleter *et al* 1999) which was located within the sequence amplified by the MY09/11 primers. The primer set had been described originally as a six primer set (Kleter *et al* 1998) but modified to include another four sequences. The PCR had final concentrations of 2.0 mM $MgCl_2$, 50 mM KCl, 10 mM Tris (pH 9.0), 0.1% Titron X-100, 0.01% gelatine, 200 μM dNTP, 1.5 unit of AmpliTaq Gold DNA polymerase (Perkin Elmer) and 15 pmol of each primer. Initial denaturation at 95°C for 9 minutes followed by 40 cycles of 94°C for 30 seconds (denaturation), 52°C for 45 seconds (annealing), and 72°C for 45 seconds (extension), with an additional 5 minutes extension. The biotinylated PCR products amplified by the SPF10 primer set were detected in a microtitre-based hybridisation assay using a mixture of probes (Kleter *et al* 1998).

7.3.3. DNA Enzyme ImmunoAssay (DEIA)

This microtiter-based hybridisation assay was used to detect biotinylated PCR fragments amplified by both the SPF10 and the MY09/11 primer sets. The assay uses a mixture of HPV specific probes (Table 7.1) to capture the PCR products. Ten μl of PCR product were placed into 100μl of hybridisation buffer (150mM NaCl, 15mM sodium citrate, 0.1% Tween 20, pH 7.0) in a streptavidin-coated microtitre plate and incubated at 42°C for 30 minutes. Then each well was washed 3 times with hybridisation buffer (400μl for 30 seconds). Then the non-biotinylated complementary strands were separated by denaturation with 100μl alkaline NaOH for 5 minutes and removed by washing three times with hybridisation buffer. The next stage involved hybridisation by adding 100μl of a probe mixture containing 9

oligonucleotides labelled with digoxigenin (DIG). After incubation at 42°C for 45 minutes the wells were washed 3 times and 100μl anti-DIG alkaline phosphate added. After a further incubation at 42°C for 15 minutes, the wells were washed 5 times. Then 100μl of substrate was added, incubated at room temperature for 15 minutes and the reaction stopped with acid. The optical densities were determined at 450nm. The presence of HPV DNA is determined by comparing the optical density with those obtained by the positive and negative controls.

7.3.4. Amplification of HPV DNA from clinical samples

All 100 cervical smears were tested for the presence of HPV DNA with both primer sets. HPV DNA was amplified from 42 samples by the MY09/11 primers and 80 samples by the SPF10 primer set (Table 7.2). In 42 samples HPV DNA was detected by both primer sets and in 38 samples only the SPF10 primers detected HPV DNA (Fig. 7.1). Twelve of these 38 samples which the MY09/11 primers had not shown any detectable HPV DNA were shown to contain high risk HPV types by the LiPA (genotypes 16, 18, 31, 39, 45, 51, 52, 56 and 68). All the samples in which the MY09/11 primers detected HPV DNA were also detected by SPF10.

7.4. Specific genotyping by a reverse hybridisation assay

The biotinylated products amplified from both MY09/11 and SPF10 primers were typed using reverse hybridisation. This involved using the INNO-LiPA HPV prototype research genotype assay. This is based on a method described for HCV (Stuyver *et al* 1996) using oligonucleotide probes developed at Delft Diagnostics Laboratory (Kleter *et al* 1999).

7.4.1. Line Probe Assay (LiPA)

The probes were immobilized as parallel lines on nitrocellulose membrane strips. They were able to detect 25 different HPV genotypes and differentiate between low risk types (6, 11, 34, 40, 42, 43, 44, 53, 54, 70, 74) and high risk types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68). Ten μl of biotinylated PCR products were denatured by the addition of 10 μl alkaline NaOH for 10 minutes in individual troughs of a tray and then the LiPA strips added. All the following steps were performed automatically in an Auto-LiPA (Innogenetics, Belgium). Two ml of hybridisation buffer (45 mM Na-citrate, 450 mM NaCl, 0.1% sodium dodecyl sulphate) was added and incubated at 50°C for 1 hour. The strips were washed twice in hybridisation solution. The strips were incubated in alkaline phosphate-streptavidin conjugate for 30 minutes at room temperature. After 3 washes the substrates 5-bromo-4-chloro-3-indolyphosphate (BCIP) and nitroblue tetrazolium (NTB) was added, incubated at room temperature for 30 minutes and the reaction stopped. Hybridisation was shown by a purple colour on the probe and the strips were interpreted by comparing the hybridisation pattern to those given by type specific HPV (Fig. 7.2).

7.4.2. Prevalence of HPV genotype

Twenty one different HPV genotypes were detected by the LiPA in this cohort of 100 samples, 13 of which were classed as high risk types (Table 7.3). Forty three samples contained a single HPV genotype. Twenty four of the cervical samples contained multiple infections with between 2-5 HPV genotypes being detected. LiPA did not detect a genotype in 33 samples, even though HPV DNA had been detected in 13 of these by the SPF10 PCR primer set. This is because the LiPA does not detect all low

risk types and therefore these 13 could be low risk types or new sequences not yet determined.

Overall, high risk HPV genotypes were detected in 48% (n=48) of the cervical samples, of which 21 were mixed infections (Fig. 7.3) These 21 mixed infections comprise of two groups: multiple high risk genotypes only (n=10), and a mixture of high/low risk genotypes (n=11). The most prominent high risk genotypes detected from this group of patients were type 16 (n=14), 18 (n=7), 31 (n=10) and 51 (n=10). The LiPA detected low risk HPV genotypes in 19% (n=19) of the samples, of which 3 contained mixed infections.

In the cervical samples where HPV DNA had been detected by the SPF10 primers but not by the MY09/11 primers, there was a wide range of genotypes identified (6, 11, 16, 18, 31, 39, 42, 44, 45, 51, 52, 53, 54, 56, 68, 70 and 74). Samples from this group contained 30 single and 8 mixed infections. The single infections were comprised of 8 high risk and 22 low risk genotypes. The mixed infections comprised of multiple high risk (n=1), multiple low risk only (n=2) and a mixture of high/low risk (n=5) genotypes. There was no significant correlation between any particular HPV genotype and the inability of MY09/11 to amplify it.

7.4.3. HPV genotype in different grades of cervical smear

There was an increase in the proportion of high risk HPV genotypes with the severity of the lesions (Fig.7.4). In normal or borderline dysplasia there were high risk HPV genotypes detected in less than 50% of the samples. This proportion increased to 76% in the samples from women classed with mild dysplasia and to 100% in the samples from women classed with moderate or severe dysplasia. The proportion of HPV genotypes detected in cervical smears was a mixture of low risk and high risk

HPV at the lower clinical grade, but at higher clinical grades the high risk dominated (Fig. 4). There were 13 HPV different genotypes (high and low risk) detected in the samples graded normal or borderline dysplasia (Table 7.3), while in the samples graded with severe dysplasia there was only genotypes 16, 18, 31 and 51 (all high risks) detected.

In the group of women who attended the clinic for referral or who were under regular review, 83% had HPV DNA detected in their cervical cells of which 75% were from high risk HPV genotypes. In the group of women attending after treatment, 75% had HPV DNA detected in their cervical cells of which 25% contained high risk HPV. The length of time since these women had undergone treatment varied (6 months -10 years).

7.5. Discussion

Clearly, with such a wide range of HPV genotypes it is impossible to design a primer set that is highly complementary to all. The Manos primers used in this study were the original ones described by Manos and colleagues (1989). When the Manos primer set was designed only 5 of the genital HPV genotype sequences had been reported. MY09/11 are degenerate primers and therefore a mixture of 24 unique oligonucleotide sequences. The SPF10 primer set was designed when more HPV genotype sequences were known and are not degenerate primers but a mixture of 10 primers.

In this study there were differences in the amplification ability of the two PCR systems, SPF10 and Manos. The SPF10 primer set detected HPV DNA in 80% of the samples compared to 42% by the Manos primer set. This different amplification ability could be due to specific HPV genotypes not being amplified by one of the PCR

systems. There have been reports that MY09/11 primers are less efficient at detecting certain genotypes such as HPV35 (Qu *et al* 1997), although in this study it detected HPV DNA in the single sample where it was present. In this set of samples, MY09/11 primers did not detect the HPV genotypes 42, 68, 70 and 74 as determined by the SPF10/LiPA system. This could be due to either a difference in sensitivity or specificity of the MY09/11 primers. The HPV genotypes 6, 11, 16, 18, 31, 39, 44, 45, 51, 52, 53, 54, 56 and 68 were detected in fewer samples by the MY09/11 primers compared with the SPF10 primer set (Table7.3). Since it is generally believed that consensus primer sets do not have a uniform efficiency of amplification across the HPV genotypes, this discrepancy could be due to a low viral load.

The variation in the sensitivity to individual HPV genotypes by consensus primers was due to primer design and the number and amount of each oligonucleotide sequence present. With degenerate primers the synthesis relies on the random insertion of bases. Therefore an equal proportion of each sequence cannot be guaranteed. It is possible to obtain sequences in optimal proportions by synthesising them separately and then mixing them, as is done with the SPF10 primer set. Recently the Manos primers have been modified (Gravitt *et al* 2000). The novel PGMY09/11 primer set is a mixture of defined oligonucleotide sequences targeted at the same region as the MY09/11 primers. By this modification an increased sequence complementarity to a broader range of HPV types was obtained.

Another reason for the different amplification efficiency of HPV DNA by PCR systems is the difference in the amplified product size. In certain archival samples, such as those in paraffin embedded materials, primers that produce long PCR products may have a lower amplification rate because of the damaged DNA (Baay *et al* 1996; Karlsen *et al* 1996). Therefore in archival samples it is possible that the

SPF10 PCR could have a higher amplification rate than the Manos PCR because it produces smaller PCR products (65 base pair compared to 450 base pair). In this study the samples were frozen at -70^{0}C and thawed just before testing. It is likely that there would have been little degradation of DNA and this would probably not account for the low amplification rate seen with the Manos PCR.

In 2 of the samples HPV16 was amplified by SPF10 but not by the MY09/11 primers, although it was shown that MY09/11 could detect a single copy HPV DNA in dilution series. This sensitivity result was based on purified HPV16 DNA (plasmid) which does not always reflect the sensitivity in DNA rich cells.

The LiPA was capable of detecting 25 different genotypes (14 high risk and 11 low risk) and 21 were detected in this study (Table 7.3). A wide range (n=13) of high risk HPV was detected. The predominant types were 16 (14 % of cases), 31 and 51 (10 % each) and18 (7 %). This wide range only occurred in women with low grade lesions, with the range being restricted to four types in those graded with severe dysplasia. Interestingly these were the 4 which were also the most predominant, although the small sample size restricts the interpretation.

The HPV genotypes detected in low grade lesions were a mixture of low and high risk HPV, while with progression of severity of the lesion the high risk HPV appear to dominate. A large proportion (24%) of the samples in this study had mixed infections with some having up to five different genotypes. The LiPA proved to be effective in detecting genotypes in these multiple infections which often comprised a mixture of high and low risk HPV.

The guidelines established by the British Society for Colposcopy and Cervical Pathology advises active treatment on women with severe dysplasia. In this study there is a high correlation between women with these grades of smear and the

presence of high risk HPV, an association which perhaps validates their management guideline. The group for whom HPV typing is likely to be most useful is that which includes women with smears reported as Borderline Nuclear Changes (BNC). These are required to be kept under review by colposcopy and many remain on this status for several years, undergoing repeated colposcopic examinations. A single test is unlikely to be sufficient, but the persistence of high risk HPV combined with BNC would be useful in patient management. Furthermore, it is also important to know if it is the same high risk HPV genotype that is persisting and for this reason accurate typing is necessary. The absence of high risk HPV in this group would also be useful in clinical management. The women whose cytology shows no dysplasia would normally be discharged from colposcopy. In this study 36% of women in this group had high risk HPV indicating the need to be retained under review, while the remainder could safely be discharged to primary screening.

In the post-treatment follow up group the absence of high risk HPV provides useful confirmation of cure and, therefore, discharge of the patient. It is not possible to apply any conclusions on the presence of high risk HPV in 30% of the women in this study who had been previously treated because of the considerable length of time in which some of the follow up samples were taken. The use of HPV DNA detection in the colposcopy clinic provides us with more information which could help in more accurate targeting of treatments and decisions on when to release women back to primary screening.

In conclusion, HPV DNA detection is a useful tool when used in conjunction with cytology in the early diagnosis of cervical cancer. Both the SPF10 and Manos PCRs are useful in HPV DNA detection, with the SPF10 being more sensitive and having the advantage of being a mixture of primers rather than degenerate. It is important to

know if the HPV is a high risk type and the LiPA can provide this information. Additionally, the LiPA provides the information about the individual genotype which is very useful when looking at persistence and it can also detect mixed infections.

Table 7.1. PCR oligonucleotide primers and probes for HPV

Oligonucleotide	Sequence (5'→3')	Nucleotide position
MY09	CGTCCMARRGGAWACTGATC	4227-4247
MY11	GCMCAGGGWCATAAYAATGG	4549-4530
SPF10 primers*:		
SPF1A	GCiCAGGGiCACAATAATGG	6582-6601
SPF1B	GCiCAGGGiCATAACAATGG	6582-6601
SPF1C	GCiCAGGGiCATAATAATGG	6582-6601
SPF1D	GCiCAAGGiCATAATAATGG	6582-6601
SPF2B	GTiGTATCiACAACAGTAACAAA	6624-6646
SPF2D	GTiGTATCiACTACAGTAACAAA	6624-6646
DEIA probes:		
HPVuni1A	CAiAATAATGGCATiTGTTGGC	6591-6612
HPVuni1B	CAiAACAATGGCATiTGTTGGC	6591-6612
HPVuni1C	CACAATTAATGGCATTTGTTGGGG	6591-6612
HPVuni2	CAiAATAATGGTATiTGTTGGG	6591-6612
HPVuni3	CAIAACAATGGTATiTGTTGGC	6591-6612
HPVuni4	AACAATGGTATiTGCTGG	6591-6612
HPVuni5	AACAATGGTGTTTTGCTGG	6591-6612
HPVuni6	AATAATGGCATiTGCTGG	6591-6612
HPVuni7	AACAATGGCATiTGCTGG	6591-6612

M represents A or C, W represents A or T, Y represents C or T, M represents A or G. DEIA = DNA Enzyme ImmunoAssay. i = inosine. * four of the sequences are not published.

Table 7.2. Detection of HPV DNA by PCR in cervical smears using the MY09/11 and SPF10 primer sets, showing the genotypes detected by LiPA

Cytology	Number of samples	HPV DNA detected		High risk HPV genotypes
		MY09 / 11	SPF10	
Normal	54	12 (22%)	39 (72%)	16, 18, 31, 39, 45, 51, 52, 56, 58, 66, 68
Borderline dysplasia	11	5 (45%)	10 (91%)	16, 33, 35, 58
Mild dysplasia	18	12 (71%)	17 (94%)	16, 18, 31, 39, 45, 51, 52, 56, 58, 66
Moderate dysplasia	9	6 (67%)	6 (67%)	16, 31, 33, 51, 58, 66
Severe dysplasia	8	7 (88%)	8 (100%)	16, 18, 31,51
Total	100	42 (42%)	80 (80%)	

Table 7.3. HPV genotypes detected by LiPA in the 100 cervical smears

HPV Genotype	High or low risk	Number of times each genotype detected			
		Single infections		Multiple infections	
		SPF10	MY09/11	SPF10	MY09/11
6	low	2	1	2	1
11	low	2	1	3	0
16	high	10	8	4	4
18	high	2	2	5	4
31	high	6	3	4	4
33	high	0	0	2	2
35	high	0	0	1	1
39	high	1	1	2	0
42	low	2	0	1	0
44	low	1	0	4	2
45	high	0	0	4	2
51	high	3	0	7	6
52	high	1	1	3	0
53	low	0	0	5	3
54	low	5	2	3	2
56	high	3	1	1	1
58	high	1	1	0	0
66	high	2	2	0	0
68	high	1	0	0	0
70	low	1	0	0	0
74	low	3	0	0	0

Fig. 7.1. Comparison of MY09/11 and SPF10 primer sets

		SPF10 primers	
		HPV DNA detected	HPV DNA not detected
MY09/11 (Manos)	HPV DNA detected	42	0
	HPV DNA not detected	38*	20

* 12 of these contain high risk types

Fig. 7.2. Identification of HPV genotypes by the Line Probe Assay (LiPA)
(Photograph supplied by Delft Diagnostic Laboratory BV, Delft, The Netherlands)

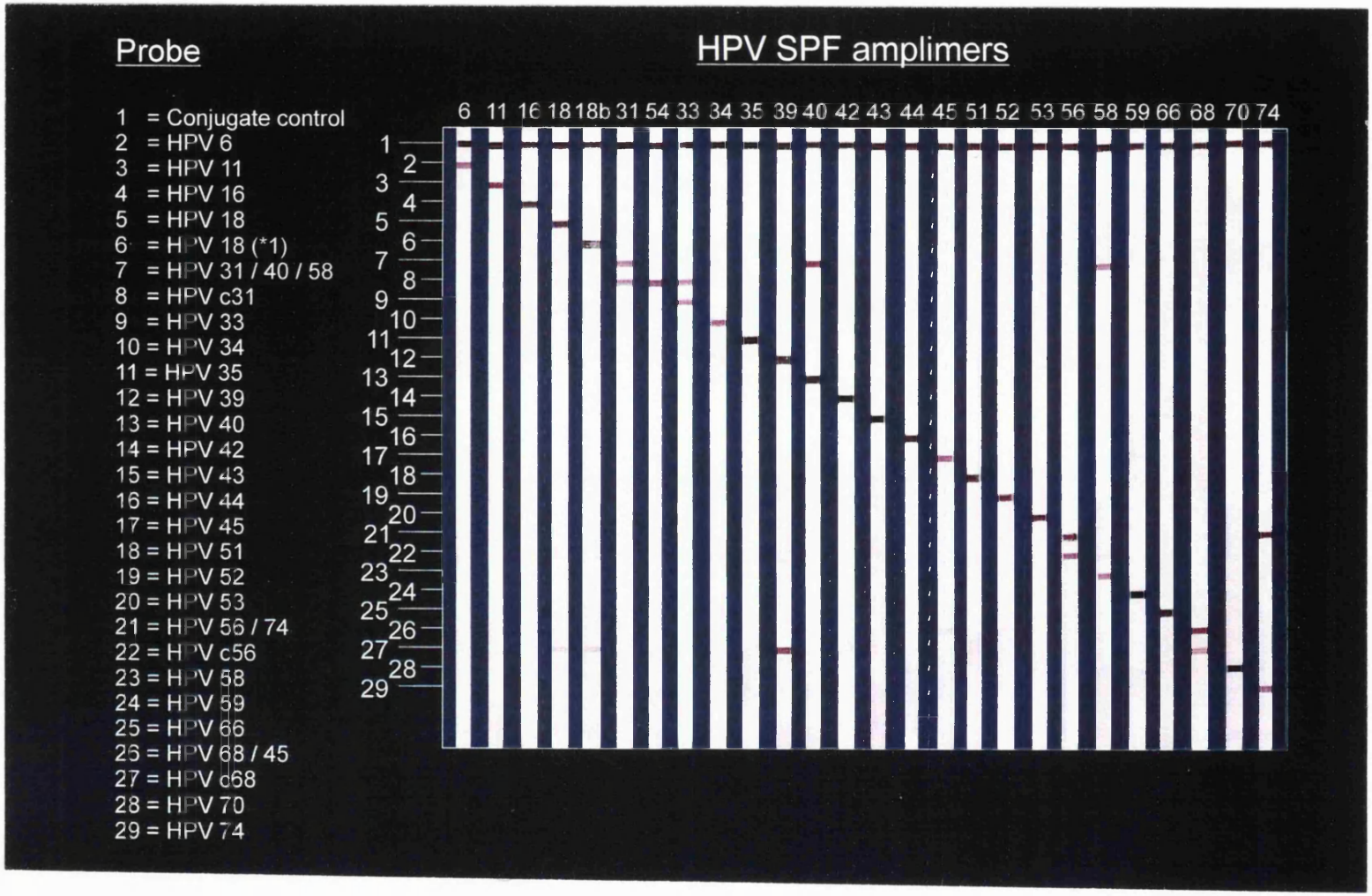


Fig. 7.3. Identification of individual and mixed HPV infections by the Line Probe Assay (LiPA)
(Photograph supplied by Delft Diagnostic Laboratory BV, Delft, The Netherlands)

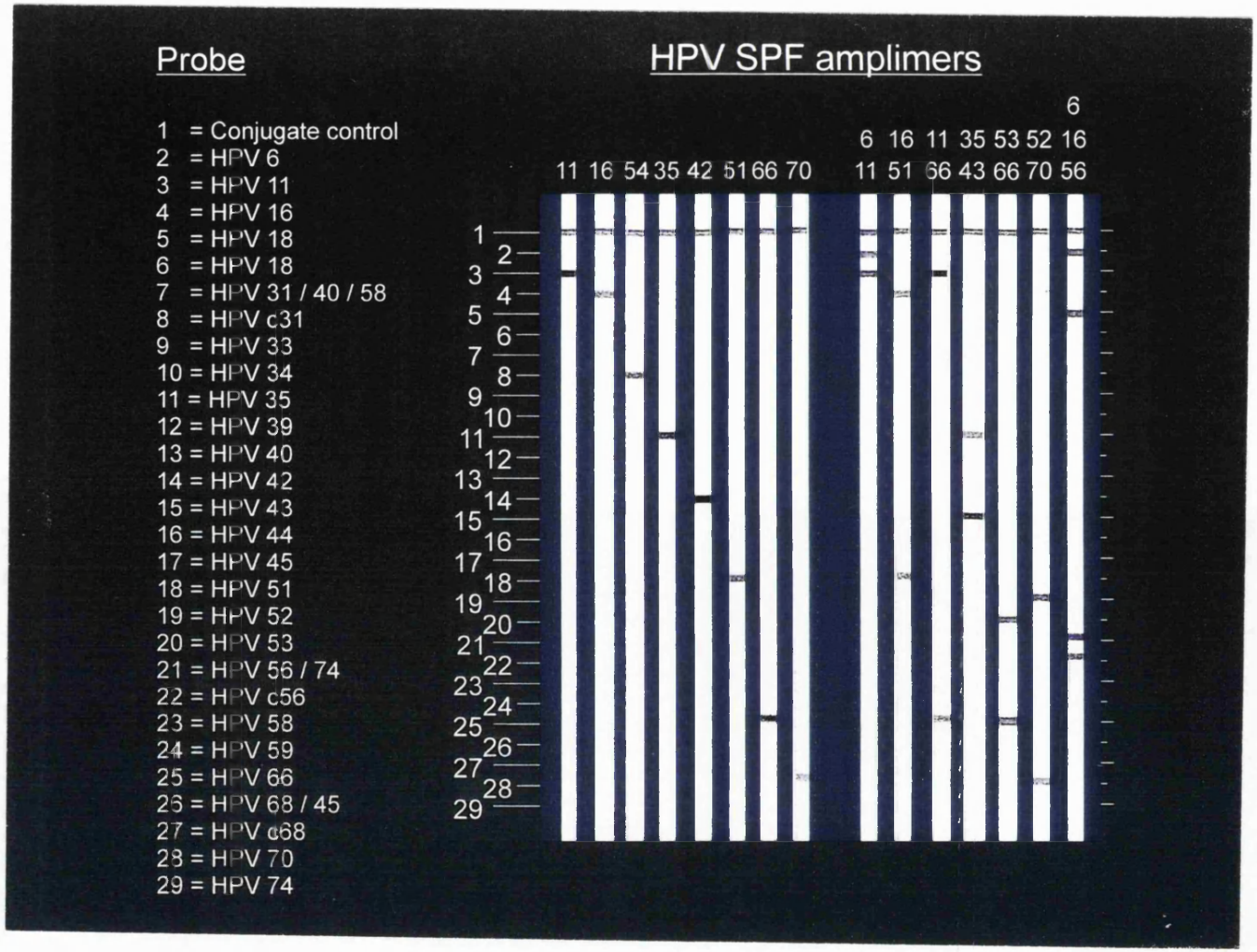
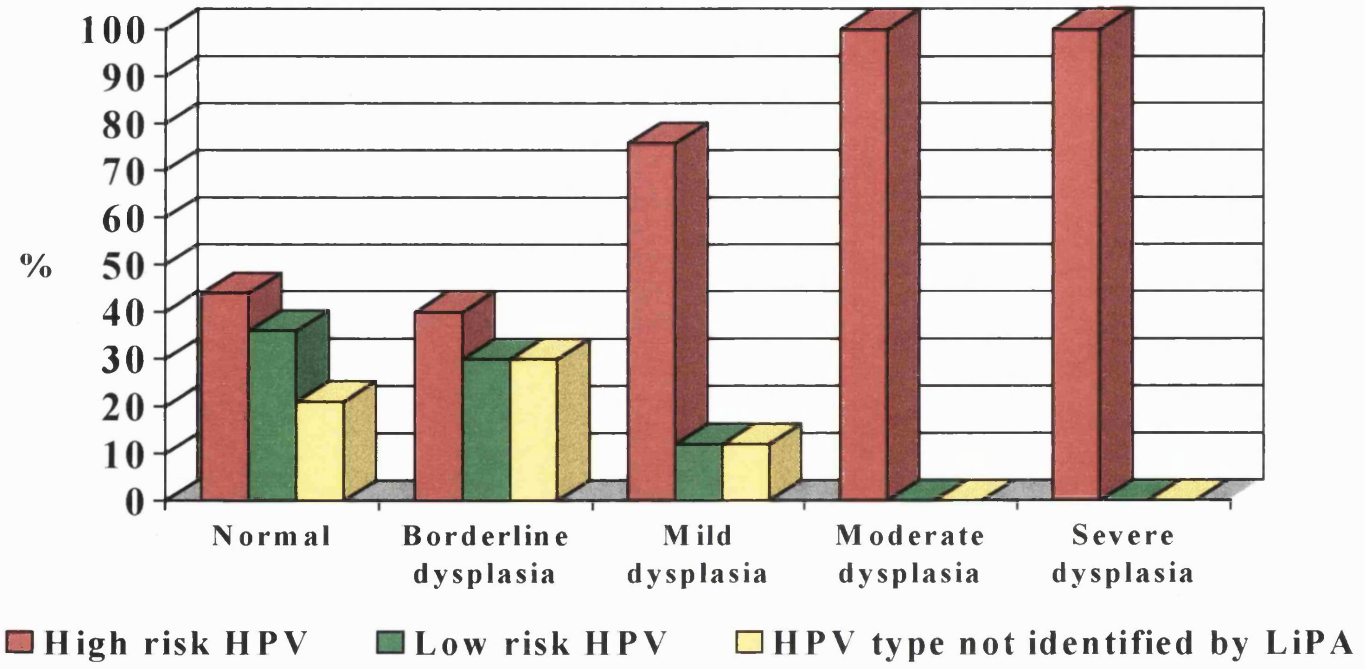


Fig.7.4. Relationship between high and low risk HPV DNA detected by SPF10/LiPA and grade of cervical smear



Chapter 8: General discussion

8.1. Development of techniques

Since some of the methods used in the study were carried out some years ago, further progress with many techniques would be expected. One particularly noticeable aspect is the availability of services that have made molecular work much quicker and easier. For example the original oligonuleotide sequences for the polyomavirus detection were prepared in the laboratory, while the later ones for sequencing and papillomavirus detection were commercially made. This also applies to DNA extraction methods where long tedious methods such as phenol chloroform extractions are now often been replaced with commercial kits. Also, the original data bases used to design the first primers had to be run overnight and those have now been replaced with almost instant searches on the world wide web. Automation has also had its impact. For example, the time consuming manual sequencing used in this study has now been replaced with automated sequencing machines. However, there are still no commercial PCR kits for the detection of polyomaviruses or papillomaviruses in clinical samples.

8.2. Polyomaviruses

8.2.1. Detection of polyomaviruses

The first part of the study on polyomaviruses aimed to design a detection system for the genomes of JCV, BKV and SV40 (chapter 3). It was originally believed that there

was a possibility that mixed polyomaviral populations would be found and so multiplexing was considered.

Two approaches to the design of the PCR were made. The first approach involved JCV, BKV and SV40 being amplified with consensus primers followed by a semi-nested PCR with specific primers to distinguish between the three polyomaviruses (Fig. 3.2). In this part of the study the emphasis was placed on primer design, therefore making it is easier to optimise the conditions to suit all targets. The initial experiments on detecting polyomaviruses in urine by the semi-nested PCR were not promising. One of the problems was that there was a drop in sensitivity when it was used as a multiplex PCR to detect both JCV and BKV in a single sample. This occured when there were large differences between the copy numbers of each virus in the sample. The virus with the least copy number was detected in lower amounts when compared to a non multiplex PCR. It is the case that if one of the amplicons anneal more efficiently with its primer it will be amplified to a greater degree than the other amplicons. It was therefore decided that this PCR needed more optimisation which would be carried out were mixed infections to be found in the CNS. It was chosen not to continue using consensus primers at this stage until we discovered what was required amplification. However, the development of the consensus primers could still prove very useful in speeding up detection in samples containing different polyomaviruses.

The study progressed with the second approach which involved three separate nested PCRs. Each PCR was designed to detect and amplify either JCV, BKV or SV40. The primer design was aimed at allowing the three sets of primers in a single assay. The nested PCR products were 150, 301 and 107 for JCV, BKV and SV40 and therefore could be distinguished by electrophoresis. Also the specificity of the PCR products

could be confirmed by enzyme digestion. Initial experiments using individual nested PCRs it was shown that they were 10-100 fold more sensitive than the semi-nested PCR. Therefore these primer sets were used for the primary aim of this study which was to detect polyomaviruses in the CNS and correlate them with disease.

The initial results did not show any evidence of BKV or SV40 in the CNS and therefore multiplex was not needed for this part of the study. However, both JCV and BKV were shed in urine and therefore a multiplex PCR would be more advantageous in this type of sample. This could be useful if reactivation of polyomaviruses in the urine correlates with disease. This would be of particular interest in post-transplantation polyomavirus infections (Boubenider *et al* 1999). Other polyomavirus multiplex PCRs have been developed (Fedele *et al* 1999) and they may have a role in the future.

8.2.2. JCV and PML

These studies (chapter 5) confirm that JCV is the aetiological agent of PML. The majority of patients with a clinical and radiological diagnosis of PML had detectable JCV in CSF. Detection of JCV DNA in any fraction of the CSF was specific for diagnosis of PML. BKV and SV40 DNA were not detected in this group of patients and no evidence was found for the association of these viruses with CNS disease.

There have been many other studies confirming that JCV is present in the brain of patients with PML (Telenti *et al* 1990; Henson *et al* 1991; Mehal *et al* 1993; Quinlivan *et al* 1992), but there is still debate over whether the virus persists in normal brain tissue. The studies in this thesis found no evidence of JCV DNA in tissue samples taken from the frontal lobe of 19 immunosuppressed patients without PML. Therefore it does not provide evidence for JCV DNA being latent in the brain,

although it is still possible that the virus is present in the brain at sites not sampled at autopsy or at levels below our threshold for detection. However, the method for JCV detection in brain tissue used in this study appears to be specific for PML.

The studies in this thesis only looked at immunsuppressed patients. Generally PML only occurs in cases of severely impaired immunocompetence as induced after organ transplantation or during AIDS. There has been one reported case of PML in a pregnant woman (Rosas *et al* 1999) and the authors speculated that a transient immunologic impairment might have reactivated a latent JC virus infection. However, the authors also concluded from this case that the criteria for this disease may need to be redefined.

8.2.3. Diagnosis of PML

Detection of JCV DNA in the CSF after PCR amplification appears to be a useful diagnostic tool, especially if combined with scanning techniques such as MRI. PCR can be used for the confirmation of PML and might obviate the need for brain biopsy. However failure to detect JCV sequences in CSF does not rule out CNS infection with this virus, a finding that has also been repeated by other workers (Telenti *et al* 1990; Gibson *et al* 1993). JCV may be present in low copy numbers in the CSF necessitating the testing of larger volumes or possibly including a concentration step in the method. Viral copy number in CSF may increase as the disease progresses and this would account for the results obtained in one patient with PML where JCV DNA was not detected in the first CSF sample but was detected in a subsequent sample taken two months later.

The results described in chapter 5 compare favourably with others on JCV DNA detection in CSF (Table 8.1). The results of the investigations described in Table 8.1,

are not consistent, but show a promising use in PML diagnosis. Over half of these studies show that the presence of JCV in CSF is specific for a diagnosis of PML. However, four of these studies show that the virus can be detected in CSF of patients without any clinical symptoms of PML, so it cannot yet be ruled out that JCV can be present in the CNS without causing disease. This lack of specificity in these four studies could also be explained by PCR cross contamination. The main problem is that the sensitivity of the PCR based techniques may lead to false positives because of contamination and, therefore, strict laboratory procedures must be carried out to avoid this. The consensus view is that detection of JCV DNA in the CSF provides useful information in a patient with neurological symptoms and, although the initial diagnosis of PML still relies on neuroimaging, it is often confirmed by the detection of JCV DNA in CSF. It must be remembered that the sensitivity for detection of JCV DNA in CSF of patients with PML varied from 72 - 100% (Table 8.1). This variance in sensitivity could be due to the methods used or PCR inhibitory substances in the CSF. Also, as suggested in this thesis, the sensitivity can be dependent on the stage of disease and so, at a more advanced stage, the increase in JCV replication improves the chances of JCV DNA recovery from the CSF. Therefore care must be taken in the interpretation of a negative PCR result and, depending on other available information, it may still be necessary to perform a brain biopsy in order to confirm JCV CNS infection.

This thesis found that JCV was shed into the urine of a proportion of immunosuppressed patients and the prevalence was higher in those patients with, than in those without, a clinical diagnosis of PML. However, this difference in detection was not significant for the specific diagnosis of PML, although the viral load being shed might have some significance.

The studies in this thesis were unable to detect the presence of JCV in peripheral blood cells from patients with or without a diagnosis of PML. Therefore it did not confirm the hypothesis that lymphocytes are an important vector for dissemination of JCV to the CNS (Major *et al* 1992). It was possible to detect human pyruvate dehydrogenase control sequences in the peripheral blood DNA indicating that the extractions had been successful. The input of the DNA into the PCR was 0.5-2.0μg representing 8.3 x 10^4 to 3.3 x 10^5 cells. Therefore JCV DNA may be present in only a small proportion of circulating peripheral blood cells and our data suggests that this sample was not useful for confirmation of PML.

8.2.4. Reactivation of JCV

It is the general view that PML is most often associated with reactivated and not primary JCV infections. However, there is conflicting evidence on possible sites of viral persistence which may be relevant to CNS disease. The possibility of the virus being latent in the brain was discussed earlier. The alternative view is that it remains in the kidney until it is reactivated (Yogo *et al* 1990). Wherever it remains latent it is believed that under certain biological pressures there is a genomic rearrangement which stimulates JCV neurotropism (Loeber and Dorries 1988).

This is further complicated by the debate on when these alterations in the regulatory region take place. One of the original hypotheses was that PML type variants are subsequently generated anew in each host from the archetype during immunosuppression (Yogo *et al* 1990; Iida *et al* 1993). Another hypothesis is that the PML types are circulating in the human population and they invade the CNS prior to disease (Elsner and Dorries 1998).

In these studies (chapter 6) analysis of the regulatory region gave rise to sequences that could be divided into four groups, A, B, C and D (Fig. 8.1). The sequences of groups A, B and C all had a closer homology to the archetype CY than to the prototype Mad1 (isolated from the brain of a PML patient). It was surprising that the regulatory region sequences isolated from PML patients would be similar to those isolated from individuals without PML (Yogo *et al* 1991). An explanation for this could be that there was a mixed population with other variants being present in lower numbers.

In one PML patient the JCV (sequence of group C) isolated from their brain was also being shed in their urine. Group A sequence is almost identical to the archetype and was only found in urine. Both group B and C sequences found in the brains of PML patients have a 13 base pair repeat. Group D sequence isolated from a patient diagnosed with PML after undergoing a bone marrow transplantation had a regulatory sequence that had not been published before.

It is hard to draw conclusions because of the small sample size. However, only regulatory region sequences similar to the archetype CY were found in this study. Only group B and C sequences, which had the 13 base pair repeat were found in brain samples from AIDS patients with PML. The same sequence being found in the brain and the urine of the same patient with PML could indicate that reactivation occurs in the kidney.

8.2.5. Treatment of PML

Progression of PML in patients with AIDS is often rapid with an average survival of four months (Berger *et al* 1987). Early diagnosis and treatment is therefore necessary for any clinical intervention to be effective. As discussed earlier there have been a

few successful attempts at treatment of PML using a number of different regimes. However there is a need for well controlled clinical trials to confirm their effectiveness. For therapeutic trials it is important to have an efficient diagnostic assay to confirm PML and a quantitative assay to monitor the viral load.

One of the original aims of the study on polyomaviruses was to develop the techniques to help monitor a controlled clinical study. The PCR described in chapter 5 proved to be very useful in detecting JCV DNA in CSF. It is routinely used in the virology department at University College London Hospital and in combination with MRI findings used to diagnose PML. A quantitative PCR (qPCR) was designed to monitor the viral load in the CSF of patients undergoing therapeutic treatment for PML. Briefly, it involved amplification by two of the primers from the nested PCR and then hybridisation of captured amplicons with an alkaline phosphatase labelled oligonucleotide probe. Quantification was achieved by measuring the intensity of light emitted by a chemiluminescent substrate (Whitby and Garson 1995). The reason this was never fully developed is as follows.

Previously the main therapeutic trial had been using cytosine arabinoside. However, a placebo-controlled study of cytosine arabinoside has failed to show any clinical benefit in patients who received the active treatment compared to those assigned to placebo (Hall *at al* 1998). Just before completion of this trial, it was found that the newly approved cidofovir diphosphate, a nucleotide analogue, suppresses JCV *in vitro* (Andrei *et al* 1997). This agent is now called cidofovir, but was originally called HPMPC [(S)-1-(3-hydroxy-2-phosphonylmethoxyproply) cytosine]. It has been reported to be associated with a single case of clinical improvement in PML (Snoeck *et al* 1996).

On this basis, under the confidentiality of a pharmaceutical company, a multicentre European study was set up. This was a controlled study involving eight countries to evaluate the safety and efficacy of cidofovir in patients diagnosed with PML. Due to the introduction of highly active antiretroviral therapy (HAART) the UK limb of this study fell in to difficulties. Very few patients enrolled on to this study and hardly any had consecutive samples and so the qPCR was not needed.

8.2.6. HAART and PML

Until the 1980s PML was a rare condition. This changed during the 1980s when it became a frequent complication of HIV infected patients. In the mid 1990s HIV therapy was transformed by the introduction of the first protease inhibitor therapy. This led to the introduction of HAART which consisted of various combinations of antiretroviral drugs including protease inhibitors. The use of the HAART regime has resulted in a dramatic improvement of life expectancy in patients with AIDS and a decline in mortality due to AIDS (Palella *et at* 1998). Also there were reports of improved survival in patients with PML who were undergoing antiretroviral therapy which included protease inhibitors (Elliot *et al* 1997; Domingo *et al* 1997).

As HAART became more widely used there where reports claiming significant gains in survival times for HIV patients with PML (Berger *et al* 1998; Albrecht *et al* 1998). It was also shown with follow up CSF analysis that JCV DNA was not detected, showing possible clearance (Cinque *et al* 1998; Miralles *et al* 1998). The application of HAART both increased the CD4 count and suppressed the HIV viral load, possibly suggesting that immune reconstitution has an effect on JCV.

It appears that AIDS patients with PML should benefit significantly from HAART. However PML still occurs in patients on HAART (Clifford *et al* 1999) and therefore

it is still necessary to address more direct modes of therapy for PML. However, the clearance of virus DNA from the CSF may have important implications for diagnosis as well as for the design of future drug trials. It would be presumed that the numbers of PML cases will remain at the same level in parts of the world where HAART has not been implicated. Also PML is still a matter of concern for immunosuppressed transplant recipients.

8.2.7. BKV and SV40 in humans

In these studies no BKV was detected in the CNS (chapter 4). From the findings of this study BKV does not appear to be associated with any specific CNS disease, but BKV-specific DNA has been reported in other studies in the brain. However, there has been increasing attention on post-transplantation polyoma infections. Both BKV and JCV can reactivate in these transplant patients and are often shed in the urine. A pilot study done with the nested PCRs described in this thesis showed that 35% of patients shed polyomaviruses (18% BKV and 21% JCV) after a bone marrow transplant (unpublished). Shedding of polyomaviruses in urine is also seen in renal allograft recipients (Howell *et al* 1999; Drachenberg *et al* 1999). This is of concern because BKV has been implicated as a cause of nephropathy in renal transplant patients (Pappo *et al* 1996; Binet *et al* 1999). The infection is difficult to treat and can lead to graft rejection (Randhawa *et al* 1999). Treatment could depend on a balance between lowering the viral load by decreasing the immunosuppressive drugs, but not to the point where there is a risk of graft rejection. Therefore it would be very useful to monitor the viral load in these patients. This could be done by monitoring BKV in blood (Nickeleit *et al* 2000) or urine. The diagnostic tests developed in this

thesis would be useful in detection of BKV DNA and could be developed further for quantification.

In these studies there was no SV40 DNA detected in the human CNS. While there is very little evidence that SV40 causes PML in humans (Stoner *et al* 1998), since this study started there have been a number of studies showing an association with various tumours in the human body. These studies report SV40 being detected in brain tumours (Suzuki *et al* 1997; Huang *et al* 1999), bone tumours (Lednicky *et al* 1997; Mendroza *et al* 1998) and mesothelioma (DeLuca *et al* 1997; Galateau-Salle *et al* 1998; Griffiths *et al* 1998; Testa *et al* 1998; Rizzo *et al* 1999). These new findings have enhanced worries about a link between SV40 and malignancy. As mentioned earlier there is always a risk of contamination in SV40 PCR because of the possible high level exposure to SV40 sequences in some laboratories. However, it would be difficult to blame PCR contamination in such a large number of studies.

Controlled studies have confirmed that there appears to be no correlation between the contaminated polio vaccine and cancer incidence rates (Strickler *et al* 1998; Olin *et al* 1998). If there is no correlation with the contaminated polio vaccine, it opens up the following three questions. Can SV40 infect both monkeys and humans? Is there transmission of SV40 from person to person? If SV40 is present in humans does it cause cancer? The case against the latter, is that the virus is not found in every tumour cell (Weggen *et al* 2000) and the amount of SV40 DNA is often very small. Therefore there is need for further investigations into the link between SV40 and human cancer.

8.3. Papillomaviruses

8.3.1. Detection of papillomaviruses

In countries which have screening programmes for cervical cancer using cervical cytology there have been significant reductions in both the incidence of and mortality from cervical cancer. However, there is still a good deal of ambiguity in the management of women with low grade abnormalities because of the poor sensitivity of cervical smears. As discussed in chapter 1 the most appropriate alternative is to use methods which detect HPV DNA. Therefore the first part of the study on papillomaviruses was aimed at detecting HPV DNA using two PCR based methods (chapter 7). Both PCR systems used consensus primers and managed to amplify HPV DNA from cells taken from the cervix at a colposcopic examination. However, there were differences in sensitivity and possible selectivity. The SPF10 primer set detected 21 HPV genotypes in 80% of the cells, while the MY09/11 detected 17 HPV types in 38% of the cells. The SPF10 primers had a number of advantages. They were designed at a time when more high risk HPV genome sequences had been determined. They amplified a smaller DNA fragment which would help in samples in which the DNA was degraded. They also had the advantage of being a mixture of primers rather than degenerate. The MY09/11 primers would benefit from the modifications recently carried out by Gravitt and colleagues (2000) which consider some of these problems. These are called PGMY09/11 and are designed in the same region as the MY09/11 primers, but with greater sequence complementarity across a broad range of HPV types. They consist of 18 sequences which are synthesised separately.

A comparison between the MY09/11 primer set and another primer set GP5/6 showed differences in the sensitivities and characteristics (Qu *et al* 1997). Therefore

differences in the characteristics of a particular consensus primer set should be considered when comparing data between studies.

8.3.2. Genotyping of papillomaviruses

The consensus primers used in this study were not able to distinguish between high and low risk HPV. Therefore a second form of analysis was needed to give information from which clinical decisions could be made. Genotyping can be done in many ways such as sequencing, the use of specific genotype probes (eg LiPA) or by restriction fragment length polymorphism. The LiPA was chosen in this study to genotype the amplified HPV DNA. The LiPA used a number of probes whose sequences lie between the SPF10 primer sequences. These probes were able to detect 14 high risk HPV and 9 low risk HPV. The LiPA detected a wide range of high risk HPV genotypes from women attending the colposcopy clinic as discussed in chapter 7. Its ability to detect individual genotypes is an advantage when looking at ecology and persistence, which becomes a problem in those tests which can only distinguish between low and high risk. The LiPA was able to detect mixed infections in 24% of the women. This ability to determine individual genotypes in mixed infections is also an advantage when looking for persistence. Other methods, such as sequencing after amplification with consensus primers, result in determining only the genotyping which is present in the highest numbers.

8.3.3. HPV and cervical cancer

This thesis shows that there is a high correlation between the presence of high risk HPV and women presenting with smears of higher clinical grade (chapter 7). Investigations have shown that persistent infection with high risk HPV types is

necessary for the development of lesions and the subsequent progression to cervical cancer (Nobbenhuis *et al* 1999; Wallboomers *et al* 1999). Therefore further studies are to be carried out to look at persistence of high risk HPV at the cervix, especially in women with smears reported as Borderline Nuclear Changes (BNC). Prospective clinical follow up studies have shown that there is an increased risk of future invasive cervical cancer if there is persistence of the same type of virus (Wallin *et al* 1999). Therefore it is of clinical value in not only detecting the presence of high risk HPV but also in identifying the exact genotype.

8.3.4. Diagnosis of cancer

Cervical cytology has traditionally been the main tool in prevention of cervical cancer. However, the limited sensitivity and specificity of cervical cytology for detecting cervical cancer have been the subject of considerable debate. As discussed in chapter 1 only high grade abnormalities are treated because only 14% of women with CIN I will progress to CIN 3 (Syrjanen 1996). Also a review by Oster (1993) showed that the probability of progression to cancer increases with the severity of the lesion.

To improve this situation there has been a search for new technologies. One approach has been to improve the quality of reading cervical smears. A liquid based cytology can give much better preparations that are quicker and easier to read. To remove reading errors automation has also been introduced (Bosanquet *et al* 1999). Another approach is to look for the presence of high risk HPV. This is a powerful tool in detecting those at risk of developing cervical cancer. However, this is an expensive technology and there is some debate on how to use HPV testing. The use of HPV DNA detection for screening the whole population would lead to a high positive rate

because most women clear the infection without developing cervical lesions (Hildesheim *et al* 1994; Wheeler *et al* 1996).

Therefore several strategies have been put forward usually based on a combination of cervical cytology and high risk HPV testing. One that is being put in place in University London College Hospitals is only to HPV test women who are attending the colposcopy clinic. The use of HPV DNA detection in the colposcopy clinic provides more information which could help in more accurate targeting of treatments and decisions on when to release women back to primary screening. Women with borderline smears and the absence of high risk HPV infection could be released from the colposcopy clinics so releasing valuable resources. Furthermore, it helps remove the anxiety from the women. An alternative strategy put forward by Manos and colleagues (1999) involved HPV testing all abnormal smears before they were referred to the coloposcopy clinic. Only the women with high risk HPV infections were then referred, therefore reducing the number of women attending the colposcopy clinic.

Using these strategies it has been proposed that it is possible to increase the screening interval of women with normal smears and no detectable high risk HPV. This is based on the premise that the development of cervical cancer from a mild abnormal smear takes about 13 years (Meijer *et al* 2000). However, a 'negative high risk HPV' result should be treated with caution because the detection methods can vary in sensitivity. For example the only commercial DNA detection kit, Digene Hybrid Capture II, depends on hybridisation and is not as sensitive as PCR.

A strategy to follow persistence of HPV genotypes could involve initial testing with the high risk HPV probe. Then women who have high risk HPV detected in their first smear and their subsequent smears would be genotyped to look for persistence. The

LiPA would be ideal at looking at persistence because it can pick out individual genotypes even in mixed infections.

8.3.5. Prevention of cervical cancer

The prevention of cervical cancer can be by vaccine or treatment. Both vaccines and treatments were discussed in the first chapter. With treatment the most important thing is a diagnosis at its precursory stage. Screening the population by cytology has been a very useful tool in reducing deaths due to cervical cancer, but its lack of sensitivity might lead to HPV DNA detection being used in certain higher risk groups. PCR would be the most sensitive technique for HPV detection. Also cytology needs improving so that problems due to misinterpretation are removed.

Alternatively, if it is considered that high risk HPV infection in women can potentially lead to cancer of the cervix, then the detection of high risk HPV should be used in primary health care for prevention of this disease. This would involve using some form of HPV DNA detection in primary screening and performing cytology on the positives. This approach would need to take in the following considerations. Only a minority who are infected with HPV are likely to develop clinically significant disease. Therefore how do you counsel a young woman who has normal cytology but is infected with a virus that can potentially cause a fatal disease? Also this is a sexually transmitted disease so would it be necessary to test their sexual partners for the presence of HPV?

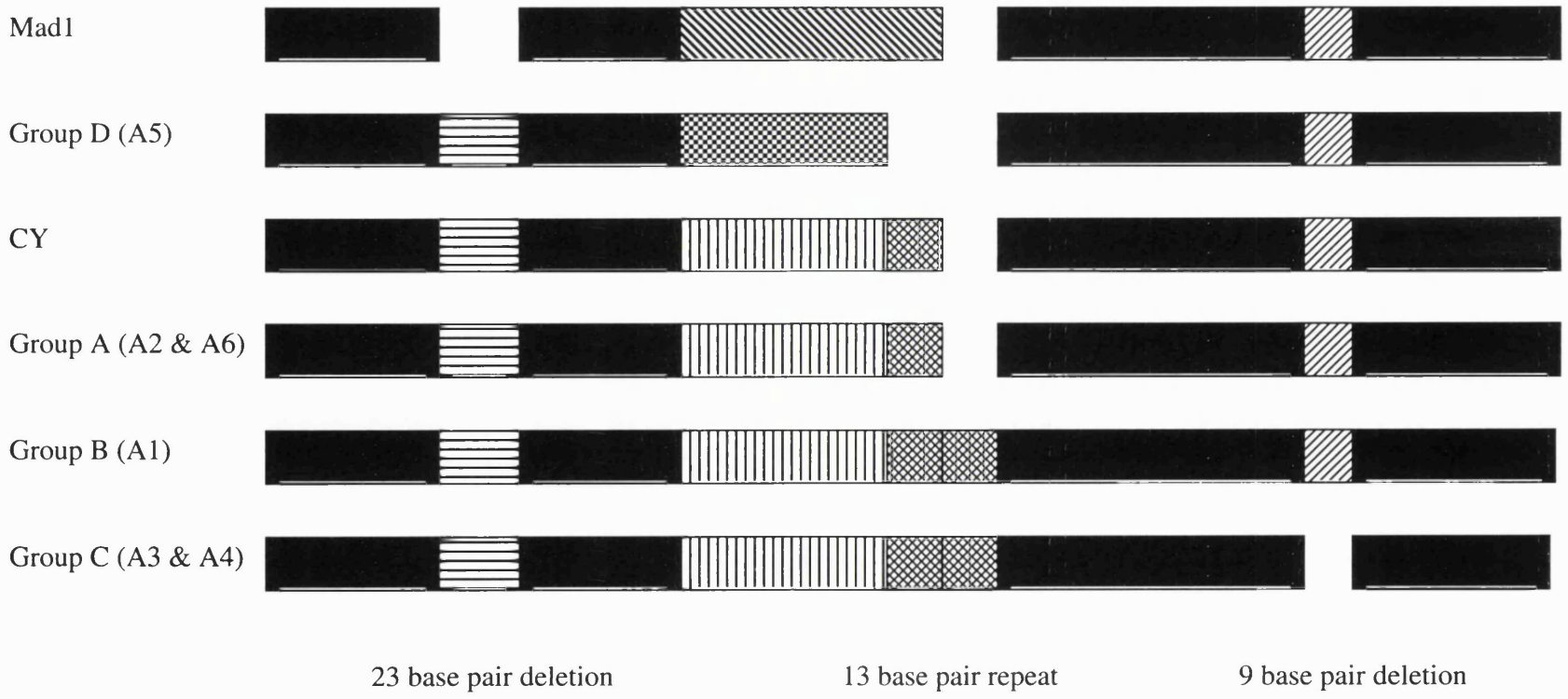
At the moment HPV detection is not perceived as a general screening tool but still plays an important role in the prevention of cervical cancer. HPV detection gives us more information to help in clinical management, especially of those women presenting with borderline smears. Although most sexually active women can be

infected with high risk HPV, most clear the infection. HPV detection can be used to monitor those who do not clear the infection and by genotyping it is possible to follow persistence of the virus. Therefore treatment can be targeted at the right women at the right time. In conclusion, whether HPV testing is used for primary health care or an additional diagnostic test, it would provide more information in the prevention of cervical cancer.

Table 8.1. Main studies showing detection of JCV DNA in CSF from individuals with and without PML

Reference	Patients with PML		Control group	
	Number detected/total	Sensitivity (%)	Number detected/total	Specificity (%)
Moret *et al* (1993)	9/9	100	0/8	100
Gibson *et al* (1993)	10/13	77	0/41	100
McGuire *et al* (1995)	24/26	92	11/130	92
Weber *et al* (1994)	23/28	82	0/82	100
Fong *et al* (1995)	17/23	74	2/48	96
Perrons *et al* (1996)	19/23	83	0/67	100
Cinque *et al* (1996)	28/39	72	1/180	99
de Luca *et al* (1996)	14/19	74	1/83	99
Hammarin *et al* (1996)	20/20	100	0/14	100

Fig. 8.1. Schematic diagram of the regulatory sequences from the four types of JCV isolated from brain and urine, the prototype Mad1 and the archetype CY



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