Molecular characterization of the first polyomavirus from a New World primate: squirrel monkey polyomavirus

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DNA samples from a variety of New World monkeys were screened by using a broad-spectrum PCR targeting the VP1 gene of polyomaviruses. This resulted in the characterization of the first polyomavirus from a New World primate. This virus naturally infects squirrel monkeys (Saimiri sp.) and is provisionally named squirrel monkey polyomavirus (SquiPyV). The complete genome of SquiPyV is 5075 bp in length, and encodes the small T and large T antigens and the three structural proteins VP1, VP2 and VP3. Interestingly, the late region also encodes a putative agnoprotein, a feature that it shares with other polyomaviruses from humans, baboons and African green monkeys. Comparison with other polyomaviruses revealed limited sequence similarity to any other polyomavirus, and phylogenetic analysis of the VP1 gene confirmed its uniqueness.

INTRODUCTION

Polyomaviruses (PyVs) are non-enveloped viruses with a circular, double-stranded DNA genome of about 5 kb. The genome consists of an early and a late coding region, and transcription is bidirectional from the origin of replication (ori), which lies within the non-coding, regulatory region. The early region encodes the viral regulatory proteins, the small and large tumour (T) antigens. The late region of all PyVs encodes the viral structural proteins, VP1, VP2 and VP3, but in some PyVs, the late region also accommodates the gene for an agnoprotein, which is located upstream of the VP2 gene.

PyVs cause infections in birds and mammals. Primate PyVs have been characterized from humans (JC, BK, KI and WU viruses) (Allander et al., 2007; Gardner et al., 1971; Gaynor et al., 2007; Padgett et al., 1971), chimpanzees (chimpanzee polyomavirus, ChPyV) (Johne et al., 2005), macaques (simian virus 40, SV40), baboons (simian virus 12, SA12), and African green monkeys (African green monkey polyomavirus; lymphotropic polyomavirus) (Sweet & Hilleman, 1960; Takemoto et al., 1982; Takemoto & Segawa, 1983; Valis et al., 1977; zur Hausen & Gissmann, 1979). Other mammalian PyVs have been characterized from cattle (bovine polyomavirus, BoPyV) (Schuurman et al., 1990) and rodents such as hamsters and mice [hamster and murine polyomaviruses; murine pneumotropic virus (MPtV)] (Graffi et al., 1968; Gross, 1953).

In humans, JC virus (JCV) and BK virus (BKV) exist as persistent infections, with high prevalences in the human population worldwide of about 80 and 90 %, respectively. Infection with JCV and BKV is asymptomatic in healthy individuals, but reactivation can occur in immunocompromised individuals, such as AIDS patients and transplant recipients, causing serious pathogenic sequelae. JCV can cause the lethal brain disease progressive multifocal leukoencephalopathy (PML), and reactivation of BKV can cause irreversible damage to the kidneys due to polyomavirus-associated nephropathy (PVAN). Recently, two additional human viruses, KI and WU viruses, have been characterized from patients with respiratory-tract infections, although their role as causative agent has not yet been confirmed (Allander et al., 2007; Gaynor et al., 2007).

The macaque PyV SV40 has transforming properties in cell culture, and also induces tumours in newborn hamsters (Eddy et al., 1962; Girardi et al., 1962). The discovery that SV40 had been a contaminant of poliovirus vaccines produced on monkey cells, and that millions of people had...
been exposed accidentally to SV40 by this route, initiated much interest in the potential correlation between SV40 infection and the occurrence of human cancers (reviewed by Butel et al., 2003; Dang-Tan et al., 2004; Elmishad et al., 2006; Poulin & DeCaprio, 2006; White et al., 2005). The zoonotic potential of SV40 is emphasized by the finding of antibodies to this virus in the blood of humans working with primates (Engels et al., 2004; Shah, 1966).

Because of the potential threats of zoonotic transfer of PyVs to humans, knowledge of PyVs infecting non-human primates is important, especially those viruses found in primate species commonly used in biomedical research. PyVs have been characterized from several species, such as macaques and baboons, but until now, no such virus has been characterized from New World monkey species used in research, such as marmosets (Callithrix sp.), tamarins (Saguinus sp.), owl monkeys (Aotus sp.) and squirrel monkeys (Saimiri sp.). In this paper, we present the complete nucleotide sequence of a PyV infecting Bolivian squirrel monkeys (Saimiri boliviensis), squirrel monkey polyomavirus (SquiPyV), and demonstrate that SquiPyV is a virus that is indigenous to squirrel monkeys. Genome analysis shows a prototypical PyV genomic structure, and phylogenetic analysis of the structural gene VP1 shows that SquiPyV is related most closely to BoPyV.

METHODS

Tissue samples and DNA isolation. Spleen samples were obtained from animals that were sent for pathological examination to the Department of Pathobiology of the Veterinary Faculty, University of Utrecht. Archival frozen blood samples from S. boliviensis were also used in this study. DNA was isolated from the spleen and blood samples by using a Puregene Blood kit (Gentra Systems).

Diagnostic VP1 PCR. DNA samples were screened by using degenerate primers for VP1 as described by Johne et al. (2005). A nested PCR was performed in a volume of 50 μl, using 1 μg DNA, 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 0.01 % BSA, 1 μM each primer, 2.5 mM MgCl₂, 200 μM each dNTP and 1.25 U AmpliTaq Gold (PE Applied Biosystems). The first amplification reaction was performed with an enzyme-activation step of 15 min at 95°C, followed by 45 amplification cycles of 94°C for 20 s, 46°C for 20 s and 72°C for 60 s. In the second amplification reaction, 2 μl PCR product of the outer PCR was used as template. This second amplification reaction consisted of enzyme activation for 15 min at 95°C, followed by 45 cycles of 15 s at 94°C, 15 s at 56°C and 20 s at 72°C.

Amplification of the SquiPyV genome. The sequence of the SquiPyV VP1 PCR fragment was used to design primers for a nested long PCR that targets the remaining part of the circular DNA genome (see Supplementary Table S1, available in JGV Online). The long PCR was performed with the Expand High Fidelity PCR system (Roche Diagnostics) in a volume of 50 μl, using 1.0 μg DNA, 1 μM each primer, 2.0 mM MgCl₂, 200 μM each dNTP and 0.75 μl Expand High Fidelity enzyme mix (3.5 U μl⁻¹). Both amplification reactions consisted of an initial denaturation step of 2 min at 95°C, followed by 10 cycles of 15 s at 94°C, 30 s at 55°C and 4 min at 68°C, then followed by 21 cycles of 15 s at 94°C, 30 s at 55°C and 4 min at 68°C, in which 5 s per cycle was added to the extension phase. The reaction was finalized with 7 min incubation at 72°C.

SquiPyV-specific diagnostic PCR. A nested set of primers was designed for a PCR assay that targets the large T antigen of SquiPyV specifically (Supplementary Table S1). The outer amplification reaction was performed in a volume of 50 μl, using 1 μg DNA, 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 0.01 % BSA, 1 μM each primer, 3.0 mM MgCl₂, 200 μM each dNTP and 1.25 U AmpliTaq Gold. The reaction mixture for the inner reaction was identical to the outer reaction mixture but, as a template, 2 μl PCR product of the first reaction was used, with 3.0 mM MgCl₂. Samples were pre-heated for 15 min at 95°C to activate the enzyme, and then cycled for 20 s at 95°C, 20 s at 55°C and 50 s at 72°C for 45 rounds of amplification.

Cloning and sequence analysis. PCR products were isolated from agarose gel by using a Zymoclean Gel DNA recovery kit (Zymo Research) and thereafter cloned in the pGEM-T Easy vector (Promega). PCR fragments were sequenced either directly or after TA cloning with an ABI PRISM BigDye Terminator v3.0 Cycle Sequencing kit on an ABI PRISM 3100 Genetic Analyzer (PE Applied Biosystems). Each fragment was sequenced with at least threefold coverage to avoid sequencing errors. Sequences were analysed by using MacVector 8.1.1 (Accelrys) and SeqMan II (DNAStar) sequence-analysis software packages.

Phylogenetic analysis. Sequence alignments were made by using MacVector 8.1.1. and Se-Al v. 2.0a11 (Rambaut, 1996). The program GapStreeze was used to remove columns that contained gaps from the alignment (Los Alamos HIV Sequence Database; http://www.hiv.lanl.gov/content/hiv-db/GAPSTREEZE/gap.html). Phylogenetic analysis was performed by using PAUP version 4.0b10 (Swofford, 2002).

RESULTS

Detection of PyVs in New World monkeys

DNA was extracted from spleen tissue or frozen blood of various New World monkey species. A selection of samples was made on the basis of the New World monkey phylogeny (Schneider, 2000) and care was taken that representatives of all recognized New World monkey families (Atelidae, Pitheciidae and Cebidae) and most subfamilies were included in our selection (Table 1). All tissues were collected during routine pathological examination of zoo animals that had died due to different clinical causes. However, none exhibited disease symptoms that led us to suspect a PyV-associated aetiology.

Forty-four samples were screened for the presence of PyV sequences by using degenerate primers targeted to the VP1 structural gene in a broad-spectrum PCR assay (Johne et al., 2005). Upon analysis on agarose gel, only one sample, originating from Bolivian squirrel monkey Squi0106, showed a band of approximately 250 bp. The band was excised from the gel and the fragment was purified. Sequence analysis of the PCR fragment, followed by BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST/), confirmed that this animal had been infected with a PyV. The nucleotide BLAST (BLASTN) search gave the highest score with the MPTv strain Kilham VP1 gene (48 of 53 nt; 90 % identity). MPTv also scored best in the protein BLAST search (54 of 85 identical amino acids; 63 % identity), followed by BoPyV and ChPyV. Thus, squirrel monkey Squi0106 was
infected with a PyV that was distinct from all mammalian and avian PyV sequences deposited in GenBank.

**Amplification and sequence analysis of the complete PyV genome**

To characterize this novel virus further, attempts were made to amplify the remaining part of the genome. We designed a set of primers on the sequence of the initial VP1 PCR product (Supplementary Table S1). The primers were directed outwards from the VP1 fragment and thus were designed to amplify, in a nested long PCR, the remainder of the circular PyV genome. Following amplification, a band of approximately 5 kb was visualized on an agarose gel. After purification from the gel, the fragment was cloned by TA cloning in the pGEM-T easy vector and sequenced by using a primer-walking strategy.

The sequence represented a nearly full-length genome sequence, lacking only a short VP1 sequence. A contig representing the complete genome of this PyV was obtained when the initial 0.25 kb VP1 sequence was aligned with the 5 kb fragment. The deduced consensus sequence of the viral genome was 5075 bp long.

Examination of putative open reading frames (ORFs) showed that the genome of SquiPyV has a typical PyV structure (Fig. 1). The location of the ORFs on the viral genome and the characteristics of the deduced amino acid sequences are listed in Table 2. SquiPyV contains an early region with an ORF for the small T antigen, and accommodates a large T antigen-encoding region. The splice-donor and -acceptor sites, necessary to create the large T antigen ORF from the early-region mRNA, were

<table>
<thead>
<tr>
<th>Family (subfamily)</th>
<th>Species</th>
<th>Common name</th>
<th>n*</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
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<tr>
<td>Atelinae</td>
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<td>Brown-headed spider monkey</td>
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<td>Ateles sp.</td>
<td>Spider monkey</td>
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<tr>
<td><strong>Pithecidae</strong></td>
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<tr>
<td><strong>Cebidae</strong></td>
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<td></td>
<td></td>
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<tr>
<td>Cebinae</td>
<td>Cebus apella</td>
<td>Tufted capuchin</td>
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<td></td>
<td>Saimiri boliviensis</td>
<td>Bolivian squirrel monkey</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Saimiri sp.</td>
<td>Squirrel monkey sp.</td>
<td>2</td>
</tr>
<tr>
<td>Aotinae</td>
<td>Aotus trivirgatus</td>
<td>Northern grey-necked owl monkey</td>
<td>2</td>
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<td>Callitrichinae</td>
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<td>Geoffroy’s tufted-earred marmoset</td>
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<td>2</td>
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<tr>
<td></td>
<td>Callithrix sp.</td>
<td>Marmoset sp.</td>
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<tr>
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<tr>
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<td>Saguinus imperator</td>
<td>Emperor tamarin</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Saguinus labiatus</td>
<td>Red-bellied tamarin</td>
<td>2</td>
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<td></td>
<td>Saguinus oedipus</td>
<td>Cotton-top tamarin</td>
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</table>

*n*Number of individuals.
determined by using the splice-site prediction program NNSPLICE version 0.9 (http://www.fruitfly.org/seq_tools/splice.html) (Reese et al., 1997). When using the splice-donor site AGAAGAG GTTGTAA (nt 4845–4831) and splice-acceptor site GTAAGCTTTTCTGTTTTTT AGATT-CCTACTTATGGTTCTGC (nt 4581–4542), the early-region mRNA is spliced into a large T ORF encoding a protein of 655 aa. No evidence was found by using this program for the existence of a middle T-encoding region.

The late region contains the ORFs for the structural proteins VP1, VP2 and VP3. The VP2/3 and VP1 ORFs have lengths of 999 and 1074 bp, respectively, which agrees with the length of the structural proteins from other PyVs. The VP3 ORF encodes a VP3 protein that is relatively small compared with other VP3 proteins (207 aa). The difference in length is mainly due to the fact that the deduced SquiPyV VP2/3 proteins, like murine PyV VP2/3, terminate almost immediately behind the nuclear-localization signal and therefore do not contain the DNA-binding domain present in other primate and avian PyV VP2/3 proteins (Fig. 2).

Characterization of a putative agnoprotein ORF

The primate PyVs JCV, BKV, SV40 and SA12 all encode a sixth protein, the agnoprotein, which facilitates the localization of VP1 and enhances cell-to-cell spread. The agnogenes of these viruses all encode highly basic proteins (pI 10.6) of 62–72 aa (Jay et al., 1981). The agnoproteins have a high degree of sequence conservation in the N-terminal and central domains (Khalili et al., 2005). We analysed the region 5’-proximal to the VP2 gene for an additional small ORF, which could encode a protein with similar characteristics. An ORF encoding a highly basic (pI 10.09) protein of 70 aa was located in the early region at nt 504–716, just upstream of the start of the VP2 gene (Table 2). Despite its resemblance in size and pI, comparison of the agnoproteins from the primate viruses with this hypothetical gene showed only a relatively limited match. Amino acid similarity scores were 31.5, 38.9, 39.2 and 39.7% with the agnoproteins of SV40, BKV, JCV and SA12, respectively. In Fig. 3, the alignment of the hypothetical SquiPyV agnoprotein with the other primate proteins is shown.

The SquiPyV regulatory region

The most variable region of the PyV genome is the non-coding regulatory region. The regulatory region of SquiPyV is situated between the small T and VP2/3 genes on the viral genome (Fig. 4). Several sequences that are characteristic for PyV regulatory regions can also be recognized on the SquiPyV genome. The minimum sequence of the origin of replication (ori) (Deyerle et al., 1989) includes a 15 bp palindromic sequence (GAGGCTTcAAGCCTC), three T antigen-binding motifs (GAGGC) and an A/T-rich stretch containing three TATA box sequences (nt 72–101). Two TATA boxes are directed forward and may involve transcription of late genes; the other has a reversed orientation. Two additional T antigen-binding motifs that are both in the reversed orientation are present 5’ of ori (nt 31–35 and 42–46), and may function in the regulation of early mRNA synthesis. Other alleged TATA boxes are found downstream of ori at nt 281–289, 319–325 and 341–348.

The regulatory regions of primate PyVs contain large, repeated sequences and multiple binding sites for transcription factors such as Sp1, NF-1 and AP-1 (Turner & Woodworth, 2001). No large, repeated sequences are present in the regulatory region of SquiPyV, but a short, repeated sequence of 12 bp is found at nt 417–440. In addition, a number of transcription factor-binding sites are present in the regulatory region of SquiPyV (Fig. 4).

### Table 2. Proteins encoded by SquiPyV

<table>
<thead>
<tr>
<th>Protein</th>
<th>Coding region (nt)</th>
<th>Length (aa)</th>
<th>Molecular mass (kDa)</th>
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<tr>
<td>VP1</td>
<td>1718–2791</td>
<td>357</td>
<td>39.9</td>
</tr>
<tr>
<td>VP2</td>
<td>778–1776</td>
<td>332</td>
<td>36.6</td>
</tr>
<tr>
<td>VP3</td>
<td>1153–1776</td>
<td>207</td>
<td>23.9</td>
</tr>
<tr>
<td>Small T antigen</td>
<td>4584–5075</td>
<td>163</td>
<td>19.0</td>
</tr>
<tr>
<td>Large T antigen</td>
<td>4839–5075, 2831–4561</td>
<td>655</td>
<td>75.1</td>
</tr>
<tr>
<td>Agnoprotein</td>
<td>504–716</td>
<td>70</td>
<td>7.8</td>
</tr>
</tbody>
</table>

Fig. 2. Alignment of the C-terminal end of VP3 from SquiPyV and other mammalian PyVs. Amino acid similarities and identities are shaded dark grey and light grey, respectively. Boxes indicate conserved domains. NLS, Nuclear-localization signal; DBD, DNA-binding domain.
Prevalence of SquiPyV infection in squirrel monkeys

To assess the prevalence of SquiPyV infection of squirrel monkeys, we designed PCR primers that targeted the SquiPyV large T antigen region specifically (see Supplementary Table S1, available in JGV Online). We reanalysed the spleen DNA samples of squirrel monkeys with this nested set of primers. The animals were unrelated and had been housed in different institutes. Five of 10 samples from *Saimiri* sp. tested positive in this assay (data not shown). Sequence analysis of the purified PCR fragments confirmed infection with SquiPyV in all five animals. Alignment of the sequences with that derived from animal Squi0106 revealed one nucleotide difference in the large T fragment of a single animal, suggesting that squirrel monkeys from different sources are infected with a highly similar PyV (see Supplementary Fig. S1, available in JGV Online). No positive results were obtained when we analysed the DNA samples from other New World monkey species by using the SquiPyV-specific PCR primers.

Phylogenetic relationship of SquiPyV to other PyVs

A phylogenetic analysis using the VP1 genes from avian and mammalian PyVs was done in order to evaluate the evolutionary relationships between them (Fig. 5). VP1 was selected because this allowed us to incorporate ChPyV in our analysis. Gaps were removed by using the GapStreeze program with the option to delete gaps in triplets to preserve codons. The optimal evolutionary model to analyse this dataset was estimated by using MODELTREE v. 3.7 (Posada & Crandall, 1998). The optimal maximum-likelihood tree was determined by using the GTR+I+Γ model and a heuristic search using the nearest-neighbour interchange followed by the tree bisection–reconnection branch-swapping algorithm. Neighbour-joining bootstrap analysis for 1000 replicates was performed by using the maximum-likelihood settings.

In the tree, all avian PyVs (finch, goose, crow and avian PyVs) form a branch separate from the mammalian PyVs. The mammalian PyVs are subdivided in two lineages. One well-supported lineage includes the four human viruses and those characterized from baboons (SA12) and macaques (SV40) (85 % bootstrap support). The two recently discovered human viruses KI and WU, although related, form a distinct subcluster within this lineage (100 % bootstrap support). SquiPyV lies on the other branch, together with the remaining mammalian PyVs. Bootstrap analysis supported only the small clusters formed by the rodent viruses and those isolated from African green monkeys (87 and 100 % bootstrap support).
respectively). SquiPyV, but also viruses from chimpanzees and cattle, form separate lineages within the group of PyVs found in mammals.

**DISCUSSION**

Research on PyVs has focused primarily on three viruses found in humans, JCV, BKV and SV40, because of their ability to cause serious diseases or their tumorigenic potential. Two recently characterized human PyVs may be the causative agents of acute respiratory infections, but several other, seemingly non-pathogenic PyVs have been described from non-human primates (Cantalupo et al., 2005; Johne et al., 2005; Pawlita et al., 1985; Takemoto et al., 1982). We have focused our search for novel primate PyVs on New World primates, with an emphasis on species used in biomedical research. This effort resulted in the characterization of the first PyV isolated from a New World monkey host, the squirrel monkey.

SquiPyV has a typical PyV genome organization, including a small ORF directly 5' of the VP2/VP3 gene that could encode an agnoprotein. The possession of an agnogene is characteristic for the JCV/BKV/SA12/SV40 subgroup of primate viruses (Khalili et al., 2005). The SquiPyV agnoprotein has limited sequence similarity to the other primate agnoproteins, but it meets the general characteristics of agnoproteins, as it is highly basic and has a length of 70 aa (Jay et al., 1981). In contrast, the supposed agnoproteins from BoPyV and budgerigar fledgling disease virus meet none of these characteristics, implying that this small ORF may well encode a primate agnoprotein. SquiPyV is genetically related most closely to BoPyV and is separated from the JCV/BKV/SA12/SV40 subcluster. The possession of an agnogene is not limited to specific lineages in the tree, but instead it is present in genomes of distantly related PyVs. One could hypothesize that agnogenes are remnants of the ancestral viruses that are conserved in these specific, but not necessarily closely related, PyVs.

Primate PyVs show a remarkable degree of sequence variation. Indeed, BLASTN analysis of SquiPyV genes presented only very limited identity over short stretches with other PyV sequences deposited in GenBank. Only when a protein BLAST search was performed did SquiPyV proteins show moderate identities (50–55%) with other PyVs, suggesting that functional constraints on the proteins have limited evolution, while allowing abundant synonymous mutations in the viral genome. The detection of novel PyVs is accordingly hampered by their genomic variation. Serological detection assays can make use of the cross-reactivity of antibodies induced to a specific, but unidentified, PyV. However, PCR-based methods to characterize viruses molecularly are intrinsically limited to the knowledge of already-known viruses. The broad-spectrum PCR primers used to detect SquiPyV were designed by Johne et al. (2005) and were based on an alignment of VP1 genes from both avian and mammalian viruses. Despite this, we could only detect the novel virus in animal Squi0106, whereas SquiPyV in the other infected squirrel monkeys was detected only after the use of

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**Fig. 5.** Maximum-likelihood tree showing the relationships of avian and mammalian PyVs, based on analysis of the VP1 genes. SquiPyV is accentuated in bold. Numbers on branches are the result of 1000 bootstrap samplings. GenBank accession numbers are indicated. Bar, 0.1 nucleotide replacements per site.
SquiPyV-specific large T primers. All large T PCR fragments obtained from various animals were highly similar (see Supplementary Fig. S1, available in JGV Online). This points to a limited inter-animal variability of the virus, and indicates that the broad-spectrum VP1 PCR assay lacks sensitivity.

SquiPyV is a PyV that infects squirrel monkeys naturally and does not represent an isolated case of interspecies transmission. Use of PCR assays to monitor the incidence of SquiPyV infections in squirrel monkeys inherently leads to an underestimation compared with serological tests, but despite this, a 50% infection rate was found. This is in line with serosurveys of other PyV infections in primates, where seropositivity rates exceed 50% (Braun et al., 1980; Ichikawa et al., 1987; Valis et al., 1977).

The AIDS epidemic of the last three decades, the increasing number of tissue transplantations and the development of drugs that specifically target components of the immune system have placed a focus on PyVs and their pathogenic potential due to reactivation (Khalili et al., 2007; Owczarczyk et al., 2007; Yousry et al., 2006). Non-human primates, infected either naturally or experimentally with PyVs, may be valuable animal models to investigate processes of viral reactivation that can lead to PML or PVAN. Zaragoza et al. (2005) investigated the use of squirrel monkeys as an animal model for human PyV infection by inoculating them experimentally with BKV and SV40. The discovery of a native SquiPyV emphasizes the necessity to use animals with a well-described viral status. Currently, the natural occurrence of PyVs in non-human primates used in animal research has scarcely been investigated. However, the intended use of primates for PyV research and, more generally, the potential zoonotic hazard of working with non-human primates that are infected naturally with PyVs requires the development of screening tools for PyV infection and research regarding the biology of PyVs. This is the first article reporting the natural occurrence of a PyV in squirrel monkeys, and represents a step towards characterization of PyVs in non-human primates used in biomedical research.

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REFERENCES


