Identification of a novel polyomavirus from vervet monkeys in Zambia

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To examine polyomavirus (PyV) infection in wildlife, we investigated the presence of PyVs in Zambia with permission from the Zambia Wildlife Authority. We analysed 200 DNA samples from the spleens and kidneys (n=100 each) of yellow baboons and vervet monkeys (VMs) (n=50 each). We detected seven PyV genome fragments in 200 DNA samples using a nested broad-spectrum PCR method, and identified five full-length viral genomes using an inverse PCR method. Phylogenetic analysis of virally encoded proteins revealed that four PyVs were closely related to either African green monkey PyV or simian agent 12. Only one virus detected from a VM spleen was found to be related, with relatively low nucleotide sequence identity (74 %), to the chimpanzee PyV, which shares 48 % nucleotide sequence identity with the human Merkel cell PyV identified from Merkel cell carcinoma. The obtained entire genome of this virus was 5157 bp and had large T- and small t-antigens, and VP1 and VP2 ORFs. This virus was tentatively named vervet monkey PyV 1 (VmPyV1) as a novel PyV. Comparison with other PyVs revealed that VmPyV1, like chimpanzee PyV, had a longer VP1 ORF. To examine whether the VmPyV1 genome could produce viral proteins in cultured cells, the whole genome was transfected into HEK293T cells. We detected VP1 protein expression in the transfected HEK293T cells by immunocytochemical and immunoblot analyses. Thus, we identified a novel PyV genome from VM spleen.

INTRODUCTION

Members of the family Polyomaviridae are non-enveloped viruses carrying a circular dsDNA genome of ~5000 bp and consisting of an early and a late coding region. Viral transcription is bidirectional from the origin of replication, which lies within the non-coding, regulatory region. The early coding region encodes regulatory proteins, known as tumour antigens, including a large T-antigen (TAg) and a small t-antigen (tAg), whereas the late coding region encodes the structural proteins VP1, VP2 and VP3. Some primate and human polyomaviruses (PyVs) also carry an agnoprotein gene upstream of the VP2 gene. In contrast, avian PyVs carry a VP4 gene, rather than the agnoprotein gene (Johne & Müller, 2007).

PyVs infect a broad range of birds and mammals, including humans. Eleven human PyVs have been identified to date: BK PyV (BKV; Gardner et al., 1971), JC PyV (JCV; Padgett et al., 1971), KI PyV (KIV; Allander et al., 2007), WU PyV...
(WUV; Gaynor et al., 2007), Merkel cell PyV (MCPyV; Feng et al., 2008), human PyV 6 (HPyV6) and HPyV7 (Schowalter et al., 2010), trichodysplasia spinulosa-associated PyV (TSV; van der Meijden et al., 2010), HPyV9 (Scuda et al., 2011), MW PyV (MWPyV)/HPyV10/MX PyV (MXPyV) (Buck et al., 2012; Siebrasse et al., 2012; Yu et al., 2012) and STL PyV (STLPyV; Lim et al., 2013). MWPyV, HPyV10 and MXPyV are probably different variants of a single species (Yu et al., 2012). Some of these PyVs cause subclinical infections with life-long persistence in immunocompetent hosts. In particular in host immunity-compromised AIDS patients and organ transplant recipients, the viruses can reactivate and cause diseases (Jiang et al., 2009).

The non-human primate PyVs simian agent 12 (SA12) and B-lymphotropic PyV were identified from the kidney cells of a vervet monkey (VM) and a lymphoblast cell line of an African green monkey, respectively (Cantalupo et al., 2005; Pawlita et al., 1985). Although SA12 was identified from an uninoculated VM kidney culture, neutralizing antibodies to SA12 were more detectable in baboons (101/151; 67 %) than in VMs (12/49; 24 %) (Braun et al., 1995). For this reason, the natural host of SA12 is thought to be baboons (Cantalupo et al., 2005). Simian virus 40 (SV40) was identified as a contaminant in monkey kidney cultures used to prepare the first poliovirus vaccine from the mid-1950s to 1963 (Shah & Nathanson, 1976). On the basis of transformation activity of SV40 in human cells (Pipas, 2009; Shein & Enders, 1962), it has been suggested that SV40 infection occurring through the use of contaminated vaccines may be a cause of some tumours in humans (Klein et al., 2002). Indeed, SV40 DNA has been reported in a variety of human tumours, such as ependymomas, osteosarcomas and mesotheliomas; however, the relationship between SV40 and these human tumours is still controversial (Bergsagel et al., 1992; Lednicky et al., 1995).

It is also controversial as to whether PyVs can be transmitted from non-human primates to humans and thereafter cause disease. To examine potential threats of the zoonotic transfer of PyVs between non-human primates and humans, the surveillance of PyVs in wildlife is important. In the current study, we examined PyVs in non-human primates in collaboration with the University of Zambia and with permission from the Zambia Wildlife Authority (ZAWA).

**RESULTS**

Detection of PyVs in spleens and kidneys from yellow baboons (BAs) and VMs

With permission from ZAWA, we collected spleens and kidneys (n=100 each) from 50 BAs (Papio cynocephalus) and 50 VMs (Chlorocebus pygerythrus) from the Mfuwe area in Zambia in 2009. In total, 200 DNA samples were extracted and screened for the presence of PyV sequences using a nested broad-spectrum PCR method with degenerate primers targeting the PyV VP1 region (Johne et al., 2005; Orba et al., 2011). The PCR results showed the presence of positive bands of ~250 bp in seven of the 200 DNA samples (3.5%; data not shown), comprising two from BA spleens, one from BA kidneys, three from VM spleens and one from VM kidneys (Table 1).

The PCR products were subsequently sequenced and analysed by a BLAST search, which demonstrated that six of the DNA sequences showed ~90 % nucleotide identity with the VP1 region of either AGMPyV or SA12, whereas one sample (VMS96), isolated from the spleen of a VM, showed 74 % nucleotide identity with the VP1 region of ChPyV (Deuzing et al., 2010) (Table 1). Among the 50 BAs and 50 VMs, one BA and one VM contained PyV genomes in both the spleen and kidney (Table 1).

Whole viral genome analysis

Next, we identified the whole viral genomes of these seven detected PyV fragments by an inverse PCR method using primers designed on the basis of the PCR-amplified VP1 nucleotide sequences described above. The whole PyV genome sequences were then determined and found to have sizes of ~5000 bp (Table 1). We found BS94 and BK94 to have an identical sequence of 5181 bp (identified as YbPyV2). VMS95 and VMS97 also had an identical sequence of 5055 bp (identified as VmPyV3). All these viral genomes had a typical set of PyV ORFs for the early (TAg and tAg) and late (VP1 and VP2/3) proteins, and those obtained from YbPyV2 and VMK96 (VmPyV2) carried the gene encoding the agnoprotein, whereas the other three (YbPyV1, VmPyV1 and VmPyV3) did not.

We also confirmed the whole PyV genome sequences using a different method. We designed individual primer sets facing outwards in the VP2/3 region from the obtained sequences against a different region from the initial VP1 gene product, and tried to detect PyV genomes from the DNA samples isolated from tissues using an inverse PCR method. We detected the PyV genome from each sample, purified it from an agarose gel and then directly sequenced the full genome.

Phylogenetic analysis of the five identified viral genomes

Phylogenetic trees of the PyV proteins (TAg, tAg, VP1 and VP2) constructed by the neighbour-joining method suggested that all the analysed early (TAg and tAg) and late (VP1 and VP2) proteins of YbPyV2 and VmPyV3 were closely related to those of AGMPyV and HPyV9 (Fig. 1). YbPyV2 and VmPyV2 were also found to be more closely related to SA12, BKV, JCV and SV40 than to other known PyVs. Although VmPyV1 and VmPyV2 were identified from the same animal, the viral proteins of VmPyV2 were closely related to those of SA12, whereas the viral proteins of VmPyV1 were related to those of ChPyV and MCPyV.
These results suggested that the same VM was co-infected with different PyVs. Interestingly, the VP1 region of the VmPyV1 genome shared low nucleotide identity (74%) with that of ChPyV (Table 1). We found that not only the VP1 protein but also other proteins of VmPyV1 shared low nucleotide identity with other PyVs such as SV40, MCPyV and ChPyV. According to the low identity between VmPyV1 and the other PyVs (Fig. 2a), VmPyV1 seems to be a novel virus. The VmPyV1 genome consisted of typical PyV ORFs (TAg, tAg, VP1 and VP2), but not the agnoprotein (Deuzing et al., 2010). The alignment results also suggested that VmPyV1, like ChPyV, encodes a VP1 protein with an extra 150 aa in its C-terminal tail, in contrast to other known PyVs (Deuzing et al., 2010). In summary, we identified five PyV genomes among the 200 DNA samples tested, one of which (VmPyV1) was a novel PyV. Next, we focused on further characterization of VmPyV1.

Transfection of the VmPyV1 genome

To test whether the VmPyV1 genome could produce viral proteins in cultured cells, the whole circular genome was transfected into human embryonic kidney 293 cells that express the SV40 TAg (HEK293T). At 4 days post-transfection (p.t.), the cells were harvested and examined for the presence of viral mRNAs and proteins.

To identify TAg and VP1 mRNA transcripts specific for VmPyV1 in transfected cells, TAg and VP1 primers were generated. TAg primers were designed to cover a predicted splicing site of the early mRNA (Fig. 2b, dotted line) (Johne & Müller, 2003). VmPyV1 TAg spliced mRNA transcripts and VP1-specific mRNA transcripts were both verified by reverse transcription-PCR (RT-PCR) (Fig. 3a). By RT-PCR for TAg, two closely separated PCR products were observed (~1200 and ~800 bp; Fig. 3a, left panel, RT(+), lane 2). Both bands were purified from an agarose gel and then directly sequenced. The sequence of the 1200 bp PCR product was consistent with that of the VmPyV1 genome, suggesting that this PCR product was an unspliced TAg mRNA transcript. The sequence of the 800 bp PCR product was in accordance with that of the VmPyV1 genome, suggesting that this PCR product was an unspliced TAg mRNA transcript. The sequence of the 800 bp PCR product was in accordance with the sequence of the VmPyV1 genome devoid of nt 4542–4938 (Fig. 2b, dotted line), thus suggesting that this PCR product was a spliced mRNA of VmPyV1 TAg. As HEK293T cells express the SV40 TAg (Soneoka et al., 1995), we checked the sequence alignment between SV40 TAg and the spliced mRNA and confirmed that the 800 bp product was not derived from the SV40 TAg. A PCR product was detected by RT-PCR for VP1, and the size of the product was similar to that of the PCR product from the VmPyV1 genome (~600 bp; Fig. 3a, right panel, RT(+), lanes 2 and 4). The 800 bp spliced and 600 bp mRNA transcripts were predicted to generate the viral TAg and VP1 proteins, respectively, in transfected cells.

To detect the protein expression and localization of VmPyV1 VP1 in transfected HEK293T cells, immunocytochemical
Fig. 1. Phylogenetic analysis of PyV-encoded proteins. Phylogenetic trees were constructed using the TAg, tAg, VP1 and VP2 proteins. The PyVs identified in this study are indicated by grey shading. The sequences of other reference PyVs were obtained from GenBank (abbreviations and accession numbers are given in Table S1). Phylogenetic analysis was performed by the neighbour-joining method with 1000 bootstrap replicates, with percentages indicated on the nodes. Bars, amino acid residue replacements per site.
analysis was performed using an anti-SV40 VP1 antibody (Kasamatsu & Nehorayan, 1979). We observed that the VmPyV1 VP1 protein was expressed and localized in the nuclei of some transfected cells at 4 days p.t. (Fig. 3b). To further confirm the antibody specificity and molecular mass of VmPyV1 VP1, we performed an immunoblot analysis. We were able to detect the VmPyV1 VP1 protein in transfected HEK293T cells and also found that its molecular mass was larger than that of JCV VP1 (positive control) (Fig. 3c, lanes 2 and 3). Overall, these results demonstrated that transfection of the VmPyV1 genome into HEK293T cells resulted in expression of its viral protein.

**DISCUSSION**

In the current study, five PyV genomes from wild BAs and VMs in Zambia were identified, four of which were shown to be closely related to AGMPyV and SA12 on the basis of the sequence and phylogenetic analyses of their full genomes and encoded proteins. These five PyVs were detected in the spleens and kidneys of two BAs and three VMs, among 50 BAs and 50 VMs examined. In these five positive animals, we also attempted to examine the presence of PyV genomes in other tissues, such as liver and lung; however, we failed to detect any.

On the basis of the phylogenetic analysis of viral proteins, VmPyV1 seems to be a novel PyV and is related to ChPyV and MCPyV. ChPyV has been identified in the faeces of chimpanzees, whereas MCPyV has been identified from human Merkel cell carcinoma, which is a rare but aggressive type of skin cancer (Deuzing et al., 2010; Feng et al., 2008). The genome structure of VmPyV1 comprises common ORF structures of PyVs, such as TAg, tAg, VP1 and VP2, but not the agnoprotein (Fig. 2a). Comparison
with other PyVs also revealed that VmPyV1 encodes an unusually long VP1 protein of 503 aa. Similarly, some PyVs possess a longer VP1, such as ChPyV (497 aa; Deuzing et al., 2010), California sea lion PyV (495 aa; Wellehan et al., 2011), bat PyV (472 aa; Fagrouch et al., 2012) and MCPyV (423 aa; Feng et al., 2008), whereas typical PyV VP1 proteins are ~360 aa (SV40 VP1, 364 aa, and JCV VP1, 354 aa). The alignment of VP1 proteins from VmPyV1, SV40, MCPyV and ChPyV is shown in Fig. S1 (available in JGV Online).

Interestingly, according to a previous report, in which ChPyV VP1 was expressed in yeast cells, the diameter of generated virus-like particles (~45 nm) was the same as that of typical PyV particles, even though the ChPyV VP1 was 497 aa. These results suggest that the number of amino acids is not related to the diameter of virions (Zielonka et al., 2011). However, the functions of this long C-terminal tail of VP1 are still unknown.

We were also able to detect transient mRNA expression of TaG and VP1 in HEK293T cells transfected with the whole circular VmPyV1 genome (Fig. 3a). The VP1 protein was also detectable by immunocytochemical and immunoblot analyses in transfected HEK293T cells at 4 days p.t. (Fig. 3b, c). We observed that the VP1 protein was predominantly present in the enlarged cell nuclei, and the molecular mass of VmPyV1 VP1 protein was found to be ~55 kDa (Fig. 3c, lane 2), which is larger than that of JCV (40 kDa; Fig. 3c, lane 3); this difference in mass was probably due to the presence of the extra C-terminal tail. Similarly, in VmPyV1-transfected COS-7 and Vero cells, we could detect the TaG and VP1 mRNAs as well as the VP1 protein by RT-PCR and immunocytochemistry (data not shown).

In the past few years, a number of PyVs have been identified in animals, including humans (Buck et al., 2012; Deuzing et al., 2010; Fagrouch et al., 2012; Groenewoud et al., 2010; Halami et al., 2010; Leendertz et al., 2011; Lim et al., 2013; Orba et al., 2011; Renshaw et al., 2012; Scuda et al., 2011; Siebrasse et al., 2012; Yu et al., 2012). African great apes have also been shown to be infected with PyVs that are closely related to MCPyV (Leendertz et al., 2011), which has provided evidence for the hypothesis that PyVs can be transmitted between humans and wild animals. In some parts of rural Africa, because humans and non-human primates may live in close proximity, accidental contact between people and these animals can occur (Hocking et al., 2010). Therefore, concerns must be raised regarding close contact between humans and non-human primates to avoid infection with pathogens, including PyVs. The surveillance of wildlife needs to be continued to examine the transmission possibility of infection agents.

In conclusion, we detected PyV genomes from non-human primates in Zambia and also identified a novel PyV from a VM spleen, which was designated VmPyV1.

**METHODS**

**Sample collection and DNA extraction.** Spleens and kidneys (n=100 each) were collected from 50 BAs and 50 VMs from the Mfuwe area (13° 14’ 42.00” S, 31° 38’ 54.07” E) in Zambia in 2009. DNA was extracted from these organs using a QiAamp DNA Mini kit (Qiagen) according to the manufacturer’s instructions. The mitochondrial cytochrome b gene was sequenced for species identification using forward primer 5’-GATACGAAAAACCATCCTGTTG-3’ and reverse primer 5’-GCTTCAAATTCCTGTTTACAG-3’ (Zimmer et al., 2009) for BAs, and forward primer 5’-GTATATGAAAAACCA- CCCTGTT-3’ and reverse primer 5’-GCTTCTTCTTCTGAGTTG-TCCATTAGG-3’ for VMs. This study was authorized by ZAWA.

**Detection of PyV genomes.** Extracted DNA was used to amplify PyV VP1 using degenerate primers (Johne et al., 2005). These primers are unlikely to detect the VP1 genes of KIV and WUV. A nested PCR was performed using 100 ng extracted DNA and High Fidelity PCR Master (Roche Diagnostics) in 20 μl reaction mixtures. The first round of PCR amplification was as follows: 2 min of denaturation at 95 °C, followed by 45 cycles of 94 °C for 30 s, 46 °C for 1 min and 72 °C for 1 min, with a final extension step at 72 °C for 5 min. For the second amplification reaction, 0.5 μl of the first PCR product was used as template, and the same cycling protocol was used, except that the annealing step was carried out at 56 °C.

**Sequence and phylogenetic analyses.** PCR products resolved and visualized by ethidium bromide-stained 1.5 % agarose gel electrophoresis were purified, subcloned into the pCR4-TOPO vector (Invitrogen) and sequenced using BigDye Terminator v3.0 Cycle Sequencing on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). After sequencing, a similarity search was performed with BLAST. The whole PyV genomes were obtained by inverse PCR using PrimerSTAR GXL DNA polymerase (Takara) and primers facing outwards from the initial PCR VP1 gene products. The inverse PCR cycling protocol comprised 2 min of denaturation at 94 °C, followed by 40 cycles of 98 °C for 10 s, 60 °C for 15 s and 68 °C for 5 min, with a final extension step at 68 °C for 5 min (Orba et al., 2011). Each whole PyV genome was then subcloned into the pCR4-TOPO vector and sequenced.

Amino acid sequences of the following reference viral genomes were obtained from GenBank: AGMyPV, bat PyV [BatPyV2a (AT7), BatPyV2b (R266), BatPyV2c (A504), BatPyV3a (A1055), BatPyV3a (B0454), BatPyV3b (B1130), BatPyV4a (R104) and BatPyV4b (C1109)], BKV, bovine PyV, budgerigar fledgling disease PyV, California sea lion PyV, canary PyV, ChPyV (ChPyV-Az, ChPyV-Bob and ChPyV-Ta), crow PyV, equine PyV, finch PyV, goose hemorrhagic PyV, Gorilla gorilla gorilla PyV, hamster PyV, HPyV6, HPyV7, HPyV9, HPyV10, JCV, KIV, Mastomys PyV, MCPyV, murine pneumotropic virus, murine PyV, Myotis PyV, MWPyV, MXPyV, orangutan PyV (OralPyV-Bor and OralPyV-Sum), Pan troglodytes verus PyV [PtvPyV1a (6444), PtvPyV1b (6520), PtvPyV2a (6512), PtvPyV2c (5924), PtvPyV2c (5927) and PtvPyV2c (6413)], SA12, STLPyV, SV40, squirrel monkey PyV, TSV and WUV (abbreviations and GenBank accession numbers can be found in Table S1). Multiple sequence alignments of the predicted TaG, tAg, VP1 and VP2 ORFs were carried out using MEGA5 Beta (Tamura et al., 2011). Phylogenetic analysis was performed using the neighbour-joining method with 1000 bootstrap replicates (Felsenstein, 1985; Saitou & Nei, 1987). Phylogenetic trees were also generated using MEGA5 Beta (Tamura et al., 2011).

**Cells and transfection of VmPyV1 genome.** HEK293T cells were cultured in an atmosphere of 5% CO2 at 37 °C in Dulbecco’s minimum essential medium, supplemented with 10% FBS, 2 mM L-glutamine, penicillin (100 U ml−1) and streptomycin (0.1 mg ml−1). All experiments using HEK293T cells were carried out in collagen-coated dishes (Iwaki).

The full VmPyV1 genome was subcloned into the KpnI site of the pUC19 vector (pUC19-VmPyV1), pUC19-VmPyV1 was digested with KpnI and the VmPyV1 genome was extracted from an agarose gel and
purified using a MonoFas Column (GL Science). Purified DNA was self-ligated in the presence of T4 DNA ligase (Takara) at 16 °C overnight. Subsequently, DNA (2 μg) purified using phenol/chlo-roform/isooamyl alcohol extraction was transfected into HEK293T cells with FuGENE HD (Roche Diagnostics).

**RT-PCR.** At 4 days post-transfection (p.t.) with the VmpyV1 genome, cells were lysed in TRIZol reagent (Invitrogen) for RNA isolation. Total RNA was then subjected to reverse transcription using random primers and SuperScript III reverse transcriptase (Invitrogen). To detect VmpyV1 cDNA, PCR was performed using Takara Ex Taq in a 20 μl reaction mixture. Tag cDNA was amplified using forward primer 5'-TCCACCTGCAATGGTACCTCTG-3' and reverse primer 5'-GGACCGAGTACTGCAAATAGTGGAG-3'. The PCR conditions were as follows: 2 min of denaturation at 94 °C, followed by 25 cycles of 98 °C for 10 s, 65 °C for 30 s and 72 °C for 1 min with a final extension step at 72 °C for 5 min. Similarly, to detect the late coding region for VP1 cDNA, PCR was performed using forward primer 5'-TTCCACATGTGTTGTTGGGG-3' and reverse primer 5'-TTACATGCGAGTACTGCAAATAGTGGAG-3'. PCR products visualized on ethidium bromide-stained 2.0 % agarose gels were detect the late coding region for VP1 cDNA, PCR was performed 1 min with a final extension step at 72 °C for 5 min. Similarly, to detect the late coding region for VP1 cDNA, PCR was performed using forward primer 5'-TTCCACATGTGTTGTTGGGG-3' and reverse primer 5'-TTACATGCGAGTACTGCAAATAGTGGAG-3'. Products were purified and then directly sequenced.

**Immunocytochemical and immunoblot analyses.** At 4 days p.t., cells were washed with PBS, fixd in 100 % methanol for 5 min at −30 °C and blocked with 1 % BSA in PBS with 0.5 % Triton X-100, followed by incubation with a polyclonal anti-SV40 VP1 antibody overnight at 4 °C (Kasamatsu & Nehorayan, 1979). Cells were visualized with secondary antibodies (Alexa Fluor 488-conjugated goat anti-rabbit IgG; Invitrogen) and 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Invitrogen) for 1 h at room temperature. Fluorescent images were captured and analysed using a microscope (IX70), a charge-coupled device camera (DP30BW) and DP Controller software (all from Olympus).

For immunoblot analysis, cells were harvested in RIPA buffer [10 mM Tris/HCl (pH 7.5), 5 mM EDTA, 150 mM NaCl, 10 % glycerol, 1 % Triton X-100, 1 % deoxycholic acid, 0.1 % SDS, 50 mM NaF], supplemented with Complete Protease Inhibitor Cocktail (Roche Diagnostics) (Orba et al., 2011). Cell lysates were centrifuged at 20,000 g for 4 °C for 15 min, and the resulting supernatants were subjected to SDS-PAGE and immunoblotting with the anti-SV40 VP1 antibody overnight at 4 °C. After washing the membrane with TBS containing 0.05 % Tween 20, the membrane was incubated with HRP-conjugated anti-rabbit IgG for 1 h at room temperature (Biosource International). The membrane was visualized using a VersaDoc 5000MP (Bio-Rad), and images were analysed using Quantity One software (Bio-Rad).

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