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Novel Polyomavirus Detected in the Feces of a Chimpanzee by Nested Broad-Spectrum PCR

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In order to screen for new polyomaviruses in samples derived from various animal species, degenerated PCR primer pairs were constructed. By using a nested PCR protocol, the sensitive detection of nine different polyomavirus genomes was demonstrated. The screening of field samples revealed the presence of a new polyomavirus, tentatively designated chimpanzee polyomavirus (ChPyV), in the feces of a juvenile chimpanzee (*Pan troglodytes*). Analysis of the region encoding the major capsid protein VP1 revealed a unique insertion in the EF loop of the protein and showed that ChPyV is a distinct virus related to the monkey polyomavirus B-lymphotropic polyomavirus and the human polyomavirus JC polyomavirus.

Members of the family *Polyomaviridae* are nonenveloped icosahedral viruses with a circular double-stranded DNA genome approximately 5,000 bp in size (6). Early genes encode two or three regulatory proteins, designated tumor antigens (T-Ag), and late genes encode the structural proteins VP1, VP2, and VP3 (7). With the exception of avian polyomavirus (APV) (14), polyomaviruses are highly species specific (6). Until now, 13 polyomaviruses infecting humans, monkeys, cattle, rabbits, rats, mice, hamsters, geese, and various bird species have been identified (6, 15). Most mammalian polyomaviruses cause subclinical infections with lifelong persistence in their natural nonimmunocompromised hosts (20), whereas polyomaviruses of birds are causative agents of acute disease with high mortality rates (22, 23, 26). The inoculation of mammalian polyomaviruses into newborn laboratory rodents induces multiple-tumor growth (7, 16, 35). It is still a matter of debate whether polyomaviruses of animals can be transmitted to humans and thereafter cause disease (5, 11, 29, 37).

The monkey polyomaviruses simian virus 40 (SV40), B-lymphotropic polyomavirus (LPyV), simian agent 12 (8), and baboon polyomavirus 2 (12) and bovine polyomavirus were originally identified as contaminants of tissue cultures (7). An unrecognized contamination of rhesus monkey kidney cell cultures used for the production of the Salk poliovirus vaccine from 1955 to 1963 lead to the exposure of an estimated 100 million people to SV40 (11). To avoid further risk of infection with unidentified polyomaviruses and to investigate their involvements in disease, broad-spectrum PCRs for the detection of thus far unknown polyomaviruses were established in this study.

For the identification of conserved regions, the genome sequences of 10 polyomaviruses (9, 10, 15, 17, 21, 24, 25, 27, 28, 32) were aligned and 12 primers (Table 1) with binding sites within short regions with high similarity were constructed. Three different nested broad-spectrum PCRs were performed with a PTC-200 Peltier thermal cycler (MJ Research; contributed by Bio-Rad, Munich, Germany) using 100 pmol of primers and 2.5 U of *Taq* DNA polymerase with buffer Y (PeqLab, Erlangen, Germany) in 50-μl reaction mixtures. The optimized cycling protocol included 5 min of incubation at 95°C, followed by 45 cycles each of 94°C for 30 s, 46°C for 1 min and 72°C for 1 min, and 72°C for 5 min. For nested PCR, 4 μl of the first

TABLE 1. Oligonucleotides used in polyomavirus broad-spectrum PCRs

PCR	Primer designation	Sequence[a] (5′-3′)	Product length (bp)
VP1 specific			
1st PCR	VP1-lf	CCAGACCCAACTARRAATGARAA	829–1137
	VP1-1r	AACAAGAGACACAAATNTTTCCNCC	
Nested PCR	VP1-2f	ATGAAAATGGGGTTGGCCCNCTNTGYAARG	249–273
	VP1-2r	CCCTCATAAACCCGAACYTCYTCHACYTG	
VP3-VP1 specific			
1st PCR	VP3-1f	CTCCAGGAGGTGCAMABCAAMG	562–631
	VP3-1r	TGCAATTCCAGAGGTTCNCCNCCNAC	
Nested PCR	VP3-2f	ACTGGATGCTGCCTYTAMTTYTAGG	394–459
	VP3-2r	TCAGTTTTTACAGTTACWGCYTCCCACAT	
T-Ag specific			
1st PCR	T-1f	GATGTTTCCTTTCTARRTTNAC	536–551
	T-1r	GCAAAGATCAAAAAAGCATHTGYCA	
Nested PCR	T-2f	AAATGATCTCTCAAGTTATCNARRTT	208–235
	T-2r	AAAGGTCCAGTTAATAGTGGNAARAC	

[a] B,C+G+T; H,A+C+T; M,A+C; N,A+C+G+T; R,A+G; W,A+T; Y,C+T.

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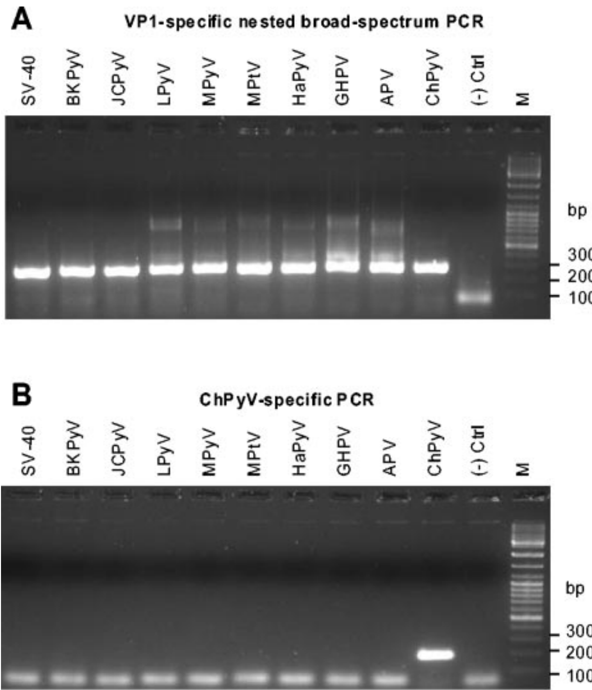

FIG. 1. Testing the VP1-specific nested broad-spectrum PCR (A) and the ChPyV-specific PCR (B) with different polyomavirus genomes. Genomic DNA of SV40, BKPyV, JCPyV, LPyV, MPyV, MPtV, HaPyV, goose hemorrhagic polyomavirus (GHPV), and APV was excised from plasmids and circularized by use of T4 DNA ligase. For the ChPyV plasmid, DNA carrying the cloned secondary PCR product of sample 12 was used. A total of 1 ng of DNA was used as the template for each PCR. PCR products were separated on ethidium bromide-stained 2% agarose gels. (−) Ctrl, negative control; M, DNA ladder mix (Fermentas, Vilnius, Lithuania).

PCR product was used as the template in a similar reaction at 95°C for 5 min, 45 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 30 s, and 72°C for 5 min. PCR products were visualized by ethidium bromide-stained 2% agarose gel electrophoresis.

The primer combinations were tested with genomic DNA of SV40, BK polyomavirus (BKPyV), JC polyomavirus (JCPyV), LPyV, murine polyomavirus (MPyV), mouse pneumotropic polyomavirus (MPtV), hamster polyomavirus (HaPyV), goose hemorrhagic polyomavirus, and APV that was rescued from plasmids and circularized by using 3 U of T4 DNA ligase (Promega, Madison, Wis.) in an overnight reaction at 16°C. A total of 1 ng of DNA was used as the template for the PCR. In all cases, secondary PCR products of the expected lengths were amplified with each of the three protocols (data not shown). The highest intensity of specific bands, however, was obtained with the VP1-specific protocol (Fig. 1A).

When this protocol was applied to 17 samples derived from different animal species with various clinical problems (data not shown), a specific PCR product was detected only in the case of sample 12 (Fig. 2A). This sample was derived from the feces of a juvenile chimpanzee (*Pan troglodytes*) that had severe diarrhea, previously tested positive for rotaviruses and *Salmonella* spp., and was kept in captivity with two other chimpanzees The amount of polyomavirus DNA in the sample was small, as a specific band was detected only after nested PCR but not after the first or the second PCR alone (Fig. 2B). After the cloning and sequencing of the PCR product, a similarity search with BLAST 2.1.3 (1) revealed a relationship with but no identity to VP1-encoding sequences of polyomaviruses. The suspected new virus was designated chimpanzee polyomavirus (ChPyV).

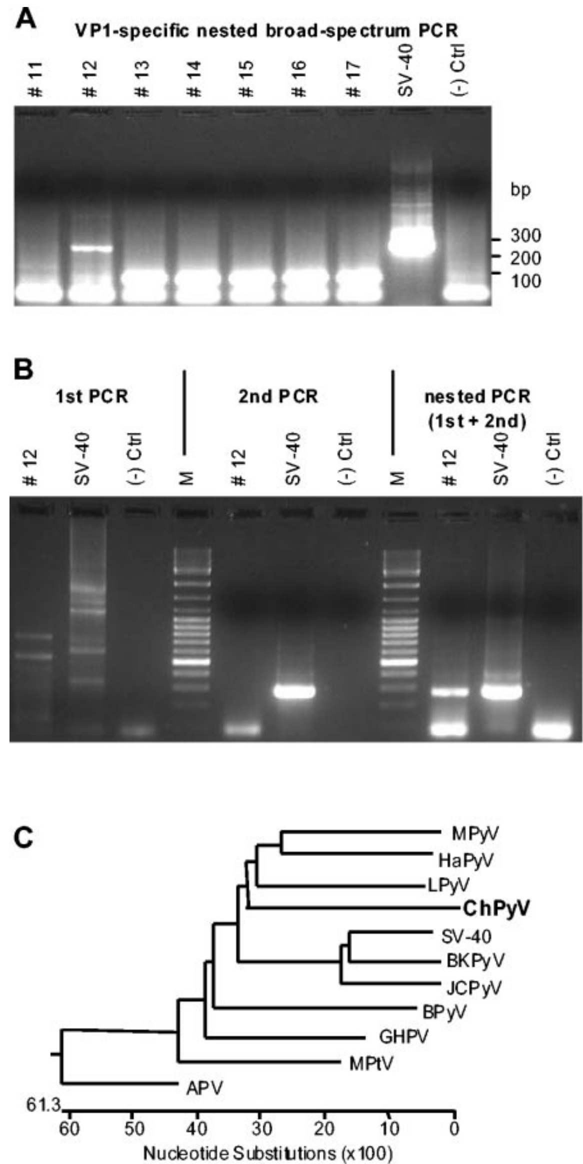

FIG. 2. Detection of DNA sequences of a new polyomavirus in clinical sample 12 derived from a chimpanzee. (A) PCR products of seven field samples (#11 to #17), SV40-infected Vero cells (SV-40), and the negative control [(−) Ctrl] were separated on ethidium bromide-stained 2% agarose gels. (B) Analysis of products amplified with primers VP1-1f and VP1-1r (1st PCR) and primers VP1-2f and VP1-2r (2nd PCR) with single PCR protocols or with the first PCR followed by the second PCR in a nested PCR protocol by ethidium bromide-stained 2% agarose gel electrophoresis. The template DNA was derived from sample 12, SV40-infected Vero cells (SV-40), or the negative control [(−)-Ctrl]. M, DNA ladder mix (Fermentas). (C) Phylogenetic relationships of the new polyomavirus (ChPyV) with 10 other polyomaviruses, based on the nucleotide sequences of the whole VP1-encoding region, aligned by the ClustalW method.

FIG. 3. Alignment of the deduced amino acid sequences of the VP1 of 11 polyomaviruses. Letters in the consensus sequence (in italics) indicate amino acids that are identical in all sequences; periods indicate positions where amino acids are not identical. Regions of VP1 forming outer loops as determined from the crystal structure of SV40 are boxed and designated BC, DE, EF, and HI loops according to reference 19.

Based on this sequence, a PCR amplifying a 195-bp fragment of the ChPyV VP1 gene was developed by using primers ChPyV-s (5′-TTTCAGCTGCTGATATCTGTGGT-3′) and ChPyV-as (5′-TCTGGGCCTGTCATAGGTTGTC-3′). The cycling profile was 95°C for 5 min, 40 cycles each of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s, and finally 72°C for 5 min. Only the cloned ChPyV sequence, and no DNA of nine other polyomaviruses (Fig. 1B), was amplified by this PCR. Testing fecal samples from the group of chimpanzees mentioned above, derived at different time points, showed that this PCR reacted positively with only one chimpanzee during the period of diarrhea (data not shown).

One hundred microliters of a dilution (1:10 in phosphate-buffered saline) of sample 12 was inoculated onto subconfluent monolayer cell cultures of human (293T and HeLa), monkey (MA104, MARC-145, and Vero), and canine (MDCK) origins, respectively. After 1 h at 37°C, the mixture was removed and the respective medium was added. After 6 days at 37°C and three cycles of freezing and thawing, cellular debris was removed by centrifugation and fresh cultures were infected with the supernatant. After five passages and an observation period of 6 weeks, however, no cytopathic changes were observed. A ChPyV-specific PCR with DNA isolated from cells from the fifth passage remained negative (data not shown).

Virus was concentrated from the supernatant of a suspension of sample 12 by ultracentrifugation. DNA extracted from the resulting pellet was analyzed by the VP3-VP1-specific and T-Ag-specific nested broad-spectrum PCRs, respectively. A polyomavirus-specific product was amplified with the VP3-VP1-specific PCR, whereas the T-Ag-specific protocol revealed only unspecific products. Primers 5′-GTTAAATGGC GCCTCCCAGGAAAAG-3′ (delineated from the VP3-VP1-specific product) and ChPyV-as amplified the 5′-region of the VP1 gene. Primers ChPyV-s and 5′-GTTTCCCAGTAGGTC TCN*AATAAA*-3′ (containing the putative polyadenylation signal for the early mRNA, which is italicized here) amplified the 3′ region of the VP1 gene. These PCRs were performed using the FastStart high-fidelity PCR kit (Roche, Mannheim, Germany). Although many other primers were tested in different combinations, no more polyomavirus-specific PCR products could be amplified from the sample.

Analysis of the assembled complete VP1-encoding nucleotide sequence (GenBank accession no. AY691168) was performed using MegAlign (DNASTAR, Madison, Wis.) with the ClustalW method (34). The lowest percentage of identity (55.8%) was found in comparisons to MPtV and MPyV, whereas LPyV and JCPyV showed the highest percentages of identity (60.5 and 61%, respectively). Phylogenetic trees established using the ClustalW (Fig. 2C), ClustalV, or Jotun Hein method consistently showed that ChPyV branches between LPyV and a group formed by SV40, BKPyV, and JCPyV.

On the deduced amino acid (aa) level (Fig. 3), the highest sequence variability is found within the BC, DE, EF, and HI loops forming the outer surface of the viral particle (19) and in the N- and C-terminal parts of VP1. The outer loops are involved in receptor binding (31) and carry antigenic epitopes together with the C-terminal part of VP1 (3, 13, 30). Phylogenetic analysis shows that these regions of ChPyV VP1 are distinct from corresponding regions of all other polyomaviruses, whereas the remaining part of ChPyV VP1 is closely related to that of LPyV (data not shown). With 395 aa residues, VP1 of ChPyV is the largest among those of the polyomaviruses, mainly due to an insertion of about 10 aa within the EF loop. This loop is located mainly at the vertices of VP1 pentamers and contributes to capsid stability by the binding of calcium ions but also extends to the outer surface of the viral capsid. A putative nuclear localization signal (RKR, aa 5 to 7) and a putative calcium binding domain (E172, D175, and D372) can be deduced from the ChPyV VP1 sequence.

Broad-spectrum PCRs were successfully utilized for the detection of new viruses, e.g., herpesviruses (36) and papillomaviruses (2). A broad-spectrum PCR was also developed for the detection of polyomaviruses (39); however, by testing 32 human tumor samples and 14 cell lines, no polyomavirus-specific sequences were amplified (38). In our investigation, nested PCR protocols developed to increase sensitivity proved to be essential, as none of the single PCRs alone was capable of detecting ChPyV DNA in sample 12. In further studies, this PCR should be applied to the screening of animal and human samples or tissue culture-derived products for polyomaviruses.

Using this approach, DNA of a novel polyomavirus was detected. Attempts to isolate virus from this sample failed, most probably due to the small amounts of virus, the poor quality of the sample, or a nonpermissive tissue culture system. ChPyV DNA could also be present in the feces of the chimpanzee due to nonproductively infected inflammatory cells which invaded the gastrointestinal tissue; hence, no infectious virus could be isolated. It remains unclear whether ChPyV infection may cause disease in chimpanzees. A distinct clinical picture could not be linked to infection with LPyV (4, 18, 33), which turned out to be most closely related to ChPyV. Further investigations should record the prevalence of ChPyV infections in chimpanzees with and without disease. With regard to the close relationship between chimpanzees and humans, the screening of human samples for ChPyV would be of special interest.

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