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Genetic characterization of the *Capra hircus* papillomavirus: A novel close-to-root artiodactyl papillomavirus

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Abstract

The healthy skin of a seven-year-old female goat (*Capra hircus*) was sampled with a cotton tip swab. Total genomic DNA was extracted from the sample and subjected to multiply primed rolling-circle amplification (RCA). Restriction analysis showed that the complete genome of a papillomavirus was amplified, measuring approximately 7600 bp in length. The *Capra hircus* papillomavirus (ChPV-1) genome was cloned from this rolling-circle amplification product, and the complete nucleotide sequence was determined. The ChPV-1 genome counts 7542 bp, and contains the typical papillomaviral open reading frames (ORFs). Based on a concatenated alignment of the E1, E2, L1 and L2 open reading frames of ChPV-1 and 54 other animal and human papillomavirus types, a neighbor-joining phylogenetic tree was constructed. In this tree ChPV-1 clusters with BPV-3, -4 and -6. Pairwise nucleotide sequence alignments of the L1 open reading frame of ChPV-1 with its closest relatives showed less than 60% similarity, placing the ChPV-1 in a novel genus.
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Keywords: Papillomavirus; Goat; *Capra hircus*; Multiply primed rolling-circle amplification; Phylogenetic analysis

1. Introduction

Papillomaviruses (PVs) are known to infect a wide variety of host-species, in which they cause proliferation of the stratified squamous epithelium of the skin or the mucosa. These PVs are responsible for a broad spectrum of genotype-specific lesions (de Villiers et al., 2004; Van Ranst et al., 1992b). In humans, the most extensively studied host, nearly 100 human PV (HPV)-types have been described based on isolates of complete genomes. The existence of an even larger number of HPV-types can be presumed based on the detection of subgenomic amplicons. An increasing investigational effort to study the infection status of most mammal and avian species has led to the characterization of new PVs in most animals. Although most discovered types infect domestic mammals, PV-types infecting a number of wild and exotic mammals and two avian species were described (de Villiers et al., 2004; Sundberg, 1987; Tachezy et al., 2002b; Terai et al., 2002). Given the possibility to detect PVs in the skin of most mammals by using a degenerate primer PCR approach (Antonsson and Hansson, 2002) and a large number of case reports of suspected PV infections in non-human vertebrates (Sundberg, 1987), the actual genotype diversity in non-human vertebrates is likely to largely exceed that of the HPVs.

The use of degenerate primers based on human cutaneous PV-sequences proved useful in the amplification of small subgenomic fragments in both human and non-human mammal hosts (Antonsson and Hansson, 2002). The drawback of this approach is that it can only detect related PV-types. To tackle this problem a multiply primed rolling-circle amplification (RCA) method was optimized which uses random hexamer primers and the ϕ29 DNA polymerase for sequence-independent amplification of papillomaviral complete genomic DNA (Rector et al., 2004b). The use of exonuclease-resistant random hexamers to prime the polymerization process eliminates the need for custom primers, so prior knowledge of the sequence to be amplified is not necessary. This allows the discovery of new PVs that are very divergent from previously characterized genotypes (Rector et al., 2004a, 2005).

Abbreviations: ChPV-1, *Capra hircus* papillomavirus type 1; E2BS*, putative E2 binding site; (H)PV, (human) papillomavirus; ORF, open reading frame; RCA, multiply primed rolling-circle amplification

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Although the first report of goat papillomatosis dates back to 1954 (Moulton, 1954; Theilen et al., 1985) and PV DNA was detected in papillomatous lesions from the mammary skin of goats using bovine PV (BPV)-type 5 DNA probes under low stringency conditions (Manni et al., 1998), to date no sequence data of this putative goat PV have been determined and its complete genomic DNA has never been amplified. We combined a non-invasive sampling technique and the optimized RCA technique to amplify a novel PV from the healthy skin of a goat.

We report the complete sequence data of a novel close-to-root papillomavirus: the *Capra hircus* PV-type 1 (ChPV-1). ChPV-1 is placed in a novel genus and is most closely related to the members of the Xi genus which hold the (bovine) PVs (BPV) that lack an E6 open reading frame (ORF) (BPV-3, BPV-4 and BPV-6) (de Villiers et al., 2004). Although ChPV-1 is most closely related to the viruses of the Xi genus, it does contain an E6 ORF.

2. Materials and methods

2.1. Origin of the samples

Samples were collected with prewetted (0.9% NaCl solution) cotton-tipped swabs that were drawn back and forth over the healthy skin of a seven-year-old female goat and then suspended in 1 ml of 0.9% NaCl solution. After the removal of the swabs, the samples were stored at −80 °C until further analysis. DNA was extracted from the samples using the QIAamp DNA Blood Mini Kit (Qiagen, Leusden, The Netherlands) following the manufacturer's protocol. This procedure yielded 36.5 μg/ml total DNA.

2.2. Multiply primed rolling-circle amplification

After a denaturation step of the circular DNA template, exonuclease-protected random hexamer primers anneal to the template DNA at multiple sites, and are extended by the ϕ29 DNA polymerase. When this DNA polymerase reaches a downstream extended primer, strand displacement synthesis occurs. These newly synthesized strands can subsequently undergo secondary priming events, thus resulting in exponential isothermal amplification. RCA generates linear, double-stranded high molecular-weight tandem-repeated copies of the template DNA (Dean et al., 2001; Nelson et al., 2002).

The multiply primed RCA reaction was performed using the TempliPhi 100 Amplification kit (Amersham Biosciences, Roosendaal, The Netherlands), following a protocol that was recently optimized for amplification of papillomaviral complete genomic DNA (Rector et al., 2004b). Ten microliters of TempliPhi sample buffer was added to 5 μl sample and subsequently heated on 95 °C for 3 min to render the DNA single stranded. We mixed 10 μl of TempliPhi reaction buffer containing salts and deoxynucleotides (dNTPs), 0.4 μl of TempliPhi enzyme mix containing the ϕ29 DNA polymerase and exonuclease-protected random hexamers in 50% glycerol, and 450 μM of extra dNTPs per sample. Ten microliters of this mix was then added to the cooled samples, and the reactions were subsequently incubated overnight (approximately 16 h) at 30 °C. Afterwards, the ϕ29 DNA polymerase was inactivated at 65 °C for 10 min.

2.3. Restriction enzyme analysis

To investigate whether papillomaviral DNA was amplified, 2 μl of the RCA product was digested with a restriction enzyme panel. After digestion, the products were run on a 0.8% agarose gel to check for the presence of a DNA band consistent with full length papillomaviral DNA (circa 8 kb), or multiple bands with sizes adding up to this length.

2.4. DNA transformation and cloning

Digestion of the RCA product with XbaI resulted in one DNA fragment of approximately 7.6 kb. Ten microliters of the RCA product was digested with 100 units of XbaI overnight and run on a 0.8% agarose gel, after which the fragment was extracted from the gel using the Geneclean kit (Qbiogene, Carlsbad, CA, USA). The 7.6 kb fragment was then ligated into a pUC18 vector that was previously cut with XbaI and dephosphorylated with Calf Intestine Alkaline Phosphatase (Fermentas, St. Leon-Rot, Germany). The ligation product was used to transform One Shot MAX Efficiency DH5α-T1R competent cells (Invitrogen, Merelbeke, Belgium), the bacteria were incubated for blue-white colony screening on agar plates containing X-gal, and white colonies were checked by XbaI digestion of miniprep DNA. One clone containing the 7.6 kb DNA fragment was selected.

2.5. DNA sequencing

The EZ::TN™ ⟨KAN-2⟩ Insertion Kit (Epicentre, Landgraaf, The Netherlands) was used to retrieve the complete viral sequence. The EZ::TN™ Insertion kit uses the Tn5 transposase to randomly insert primer binding sites and a kanamycine resistance selection marker into target DNA in vitro (Goryshin and Reznikoff, 1998).

The reaction was performed according to the manufacturer's protocol. The reaction product was used to transform One Shot MAX Efficiency DH5α-T1R competent cells (Invitrogen). Twenty-four colonies were selected and the provided primers were used to sequence the insertion clones bidirectionally from primer binding sites at the 5′- and 3′-ends of the inserted transposon. The remaining gaps in the sequence were determined by primer-walking. Sequencing was performed on an ABI Prism 3100 Genetic Analyzer (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). Chromatrogram sequencing files were inspected with Chromas 2.2 (Technelysium, Helensvale, Australia), and contigs were compiled using SeqMan II (DNASTAR, Madison, WI, USA).

2.6. DNA and protein sequence analysis

The nucleotide sequence of the ChPV-1 genome reported in this article was deposited in GenBank under accession number DQ091200.

The putative ORFs were predicted with the ORF Finder tool on the NCBI server of the National Institutes of Health (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The molecular weight of the putative proteins was calculated using the ExPASy (Expert Protein Analysis System) Compute pI/Mw tool (http://www.expasy.org/tools/pi_tool.html). Multiple nucleotide sequence alignments were constructed in DAMBE version 4.2.7 (Xia and Xie, 2001). The sequences of ChPV-1 and 54 animal and human PV-types were imported and aligned at the amino acid level using ClustalW (Thompson et al., 1994), after which the nucleotide sequences were aligned according to the aligned amino acid sequences. The unambiguously alignable regions of the E1, E2, L2, and L1 ORFs were pasted together in one compiled alignment. This alignment was used to construct a neighbor-joining phylogenetic tree in MEGA version 2.1 (Kumar et al., 2001). Bootstrap support values were determined for 10,000 iterations by the neighbor-joining method.

3. Results

3.1. The complete ChPV-1 genomic sequence

The complete nucleotide sequence of the *C. hircus* papillomavirus type 1 (ChPV-1) genome counts 7542 bp, with a GC content of 44.6% and contains the seven classical PV major open reading frames. The exact locations of the ORFs and calculated molecular weights of the putative proteins are shown in Table 1.

3.2. ChPV-1 early genes

The putative ChPV-1 E6 contains two conserved zinc-binding domains (C-X-X-C-X_{29}-C-X-X-C), separated by 31 amino acids. The E7 ORF contains one such zinc-biding domain and the conserved retinoblastoma tumor suppressor binding domain (DLTCYE).

The E1 codes for the largest ChPV-1 protein (566 amino acids), and contains the conserved ATP-binding site for the ATP-dependent helicase (GAPNTGKS) in its carboxyterminal part. ChPV-1 has, with 1572 nucleotides in the E2 ORF, a very long E2 compared to most other PVs. The E4 ORF is completely contained within the E2 gene, and in ChPV-1 an E4 start codon was identified, which is not the case in most other PVs. A high proline content (43 proline residues out 255 aa), which is typical for the E4 ORF, was noted.

3.3. ChPV-1 late genes

The late region codes for the major (L1) and minor (L2) capsid protein genes. Both L1 and L2 contain a series of arginine and lysine residues at their carboxyterminus, which is likely to function as a nuclear localization signal.

3.4. The noncoding region (NCR)

The classic noncoding region between the stop codon of L1 and the start codon of E6 counts 383 bp in ChPV-1 (nt 7175-15). PVs usually contain an E1-recognition site flanked by two E2 binding sites, for binding of an E1/E2 complex in order to activate the origin of replication. In the ChPV-1 NCR1, an E1-recognition site (E1BS, CCATGGTTGTTATCTAC) is present at nt 7466–7482. The E1BS is flanked by 2 modified putative E2-binding sites ($E2BS^*$) at nt 7436–7447 (ACC-N_6-GTG) and at nt 7502–7513 (ACT-N_6-GGT). Since the putative $E2BS^*$ are equidistant to the E1BS (17 and 19 bp respectively), these two $E2BS^*$ are probably functionally important, although it is possible that the modifications result in lower affinity binding. At its 5′-end, the NCR1 also contains a polyadenylation site (AATAAA, nt 7279–7284), upstream of a CA dinucleotide (nt 7233), and the G/T cluster, necessary for the processing of the L1 and L2 capsid mRNA transcript (Birnstiel et al., 1985). In the 3′-end, a TATA box of the E6 promotor is present at nt 7498.

3.5. Phylogenetic analysis

A neighbor-joining phylogenetic tree was constructed, based on a concatenated E1/E2/L2/L1 nucleotide sequence alignment of ChPV-1 and 54 PV-types of the different PV genera and species (Fig. 1). For this, nucleotide sequence alignments, based on the corresponding amino acid alignments, were constructed separately for the different ORFs. Regions where an unambiguous alignment could be obtained were included in one combined alignment of 2280 nucleotides. The resulting neighbor-joining phylogenetic tree clusters the PVs in the different genera, described in the new classification of PV (de Villiers et al., 2004), and the additional Rho and Sigma genus, containing the novel PVs TmPV-1 and EdPV-1 that have recently been characterized (Rector et al., 2004a, 2005). In this tree, ChPV-1 appears as a novel close-to-root papillomavirus. The ChPV-1 is the first member of a novel genus, and is according to this phylogenetic tree most closely related to the Xi genus. This genus holds the bovine

Table 1
Analysis of the open reading frames of ChPV-1

ORF	Start ORF	First ATG	Stop codon	# aa	# Nucl.	Predicted MW (kDa)
E6	1	16	433	136	420	16.06
E7	426	432	708	92	279	10.29
E1	652	691	2389	566	1701	64.57
E2	2351	2366	3935	523	1572	58.83
E4	2877	2922	3687	255	768	28.84
L2	4014	4053	5634	527	1584	56.64
L1	5606	5645	7172	509	1530	57.63

Fig. 1. Neighbor-joining phylogenetic tree, based on a 2280 bp combined concatenated E1/E2/L2/L1 nucleotide sequence alignment of ChPV-1 and 54 other animal and human type PVs. PVs included were (with their GenBank accession numbers) were BPV-1 (NC_001522); BPV-2 (NC_001521); BPV-3 (NC_004197); BPV-4 (NC_004711); BPV-5 (NC_004195); BPV-6 (NC_005350); CCPV (NC_001838); COPV (NC_001619); CPV2 (NC_006564); CRPV (NC_001541); DPV (NC_001523); EcPV-1 (NC_003748); EdPV-1 (NC_006951); EEPV (NC_001524); FcPV (NC_004068); FdPV-1 (NC_004765); HaOPV (E15111); HPV-1a (NC_001356); HPV-2a (NC_001352); HPV-4 (NC_001457); HPV-5 (NC_001531); HPV-6b (NC_000904); HPV-7 (NC_001595); HPV-9 (NC_001596); HPV-10 (NC_001576); HPV-13 (NC_001349); HPV-16 (NC_001526); HPV-18 (NC_001357); HPV-26 (NC_001583); HPV-32 (NC_001586); HPV-34 (NC_001587); HPV-41 (NC_001354); HPV-48 (NC_001690); HPV-49 (NC_001591); HPV-50 (NC_001691); HPV-53 (NC_001593); HPV-54 (NC_001676); HPV-60 (NC_001693); HPV-61 (NC_001694); HPV-63 (NC_001458); HPV-71 (AY330623); HPV-90 (NC_004104); HPV-92 (NC_004500); HPV-96 (NC_005134); MnPV (NC_001605); OvPV-1 (NC_001789); OvPV-2 (NC_001523); PCPV (NC_001678); PePV (NC_003973); PsPV-1 (NC_003348); RhPV-1 (NC_001678); ROPV (NC_002232); RPV (NC_004196); TmPV-1 (NC_006563). The numbers at the internal nodes represent the bootstrap support values, determined for 10,000 iterations with the neighbor-joining method. Only bootstrap probabilities greater than 80% are shown.

Table 2
Percentage nucleotide (amino acid) similarity of the different ChPV-1 ORFs with the ORFs of BPV-1 (GenBank accession number NC_001522), BPV-3 (NC_004197), BPV-4 (NC_004711) and BPV-6 (NC_005350)

ChPV-1 ORF	BPV-1	BPV-3	BPV-4	BPV-6
E6	39 (26)	/	/	/
E7	43 (38)	41 (37)	43 (34)	41 (38)
E1	33 (41)	33 (51)	33 (51)	33 (52)
E2	43 (31)	50 (46)	49 (45)	48 (43)
L2	40 (32)	49 (46)	50 (46)	50 (45)
L1	51 (52)	56 (57)	55 (56)	57 (59)

PVs (BPV) that lack an E6 ORF (BPV-3, BPV-4 and BPV-6) (de Villiers et al., 2004). It is therefore tempting to speculate that the necessity of an E6 ORF was lost in the common ancestor of BPV-3, -4 and -6, after this lineage had diverged from the ChPV-1 ancestor.

3.6. Sequence similarity to other artiodactyl papillomaviruses

Table 2 shows the sequence similarity between ChPV-1, its closest relatives BPV-3, -4, -6 and BPV-1 as a representative for the major artiodactyl PV genus, delta. In the L1 ORF, ChPV-1 shares less than 60% nucleotide sequence similarity with its closest relatives, confirming its classification in a novel genus (de Villiers et al., 2004).

4. Discussion

The conserved genomic organization of all known PVs provides strong evidence for a monophyletic origin. Because they use the host-cell DNA polymerase, PVs are stable and slow-evolving viruses. With a mutation rate of $0.73–1.20 \times 10^{-8}$ they evolve only 10 times faster as their eukaryotic hosts (Tachezy et al., 2002a; Van Ranst et al., 1995). Based on the absence of recombination events between different PV-types, evolution of PVs probably occurs through accumulation of point mutations. These observations combined with the worldwide distribution of PVs, which can not be explained through airborne transmission, has led to the hypothesis that PVs are ancient viruses that have co-evolved and co-speciated with their host-species during vertebrate evolution (Sundberg et al., 1997).

In order for this hypothesis of co-phylogenetic descent of PVs and their host-species to hold, PVs and their hosts have to comply with the Fahrenholz's rule. This rule states that PVs of closely related host-species should be closely related themselves, and cluster together in the PV phylogenetic tree, with dating of PV divergence largely coinciding with the host-species divergence.

Based on the appearance of the PV phylogenetic tree, in which not al PVs which infect the same host (e.g. primate PVs) are in the same phylum, it has been suggested that, instead of all modern PVs evolving out of one single primordial PV, a number of different ancient PV lineages infecting different ancestral host-species gave rise to the modern star-like appearance of the PV phylogenetic tree (Chan et al., 1995; Garcia-Vallve et al., 2005). This makes it hard (if not impossible) to use the theory of co-phylogenetic descent to calculate divergence points between members of different PV genera. Within a genus, PVs seem to comply with the Fahrenholz's rules, thus making it possible to accurately calculate divergence times of PVs that infect related hosts.

The obtained bootstrap value of 90% supports the phylogenetic relationship between ChPV-1 and the PVs of artiodactyls in the Xi genus. The divergence between the *Bovinae* and *Caprinae* hosts occurred during the Cenozoic era, and is estimated to have taken place between 17.8 and 21.4 million years ago (Kumar and Hedges, 1998). Assuming that ChPV-1 and BPV-3 diverged at the same time as their host-species, we calculated a mutation rate of $2.1–2.5 \times 10^{-8}$ nt/site per year for this PV-pair. This mutation rate is similar to the ones that were previously obtained for the PVs from the artiodactyls European elk EEPV, deer DPV, and reindeer RPV ($2.6–2.9 \times 10^{-8}$) (Van Ranst et al., 1995), the parrot PePV and the chaffinch FcPV ($0.6–0.7 \times 10^{-8}$) (Tachezy et al., 2002b), the pygmy chimpanzee PCPV-1, the common chimpanzee CCPV-1, and the human HPV-13 ($3.2–3.4 \times 10^{-8}$) (Van Ranst et al., 1992a) and for the canine COPV and feline FdPV ($0.73–0.96 \times 10^{-8}$) (Tachezy et al., 2002a).

A recent study suggested that the E6 and E7 genes gained access to the PV genome after the 'core part' (i.e. E1, E2, L2 and L1, which are present in all currently characterized PVs) was formed (Garcia-Vallve et al., 2005). However, the presence of the 7 common PV genes (E1, E2, E4, E6, E7, L2 and L1) in almost all PV genomes [the members of the Xi genus and the two avian PVs lack an E6 ORF, and only the *Phocoena spinipinnis* PV (PsPV) is lacking an identifiable E7 gene (de Villiers et al., 2004)], their conserved sequence similarity between divergent hosts, and the fact that recombination events between PVs have never been observed, raise the possibility that the six above-mentioned viruses lost the necessity for that specific gene. The results of the present study, suggesting the loss of an E6 ORF in the BPV-3, -4 and -6 ancestor after it diverged from the ChPV-1 common ancestor, support this view.

5. Conclusions

5.1. A step closer to the elucidation of papillomaviral evolution?

Recently it has been shown that healthy skin of humans harbours different (novel) PV-types, indicative of a commensalic nature for these viruses (Antonsson et al., 2000; Boxman et al., 1997). Although mostly commensalic, these viruses might be important in the aetiology of different types of lesions in (genetically) predisposed individuals (Favre et al., 1998). The identification of (novel) PV-types as aetiological agents for certain diseases might improve their treatment. A combination of a sequence-independent amplification technique and a non-invasive sampling method, like the one we used in this work makes it possible to screen the epithelia of seemingly uninfected hosts (both animal and human) for novel PVs. This allows us to tackle the two main problems of virus discovery, being the difficulty to identify lesions that do not adopt epidemic proportions and the use of hybridization-based techniques. The use of

this technique should make it possible to fill in the existing gaps in the PV phylogenetic tree and thus might help us to better understand the evolution of these ancient viruses.

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