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Molecular confirmation of an adenovirus in brushtail possums (*Trichosurus vulpecula*)

Darelle Thomson [a,*], Joanne Meers [a,1], Balázs Harrach [b]

[a] *Institute of Veterinary, Animal and Biomedical Sciences, Massey University, Private Bag 11222, Palmerston North, New Zealand*
[b] *Veterinary Medical Research Institute, Hungarian Academy of Sciences, Budapest, Hungary*

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Abstract

Partial genome characterisation of a non-cultivable marsupial adenovirus is described. Adenovirus-like particles were found by electron microscopy (EM) in the intestinal contents of brushtail possums (*Trichosurus vulpecula*) in New Zealand. Using degenerate PCR primers complementary to the most conserved genome regions of adenoviruses, the complete nucleotide sequence of the penton base gene, and partial nucleotide sequences of the DNA polymerase, hexon, and pVII genes were obtained. Phylogenetic analysis of the penton base gene strongly suggested that the brushtail possum adenovirus (candidate PoAdV-1) belongs to the recently proposed genus *Atadenovirus*. Sequence analysis of the PCR products amplified from the intestinal contents of brushtail possums originating from different geographical regions of New Zealand identified a single genotype. This is the first report of molecular confirmation of an adenovirus in a marsupial.

Keywords: Brushtail possum; Adenovirus; PCR; Phylogeny; *Atadenovirus*

Brushtail possums (*Trichosurus vulpecula*, Marsupialia) are not native to New Zealand, and were first introduced from Australia in 1837 to establish a fur industry (Pracy, 1974). They have since become New Zealand's most important vertebrate pests, with current numbers estimated at 70 million. Occupying more than 90% of the country (Cowan, 1996), they represent a serious threat to the conservation of native forests and native wildlife. They also play a role in the spread of bovine tuberculosis (Sutherland et al., 1996) with resultant losses in primary production.

Research to identify and characterise viruses of brushtail possums that could be useful for the biological control of the species began in the early 1990s. These viruses would either be pathogenic to brushtail possums, or suitable as vectors for the delivery of contraceptive antigens. An electron microscopy (EM) survey of brushtail possum intestinal contents (Rice and Wilks, 1996) revealed the presence of four types of viruses: adenoviruses, herpesviruses, coronaviruses and coronavirus-like particles. Other reports of viruses of

* Corresponding author. Tel.: +64-6-356-9099; fax: +64-6-350-5636.
E-mail address: d.m.thomson@massey.ac.nz (D. Thomson).
[1] Present address: School of Veterinary Science, University of Queensland, St Lucia 4072, Qld, Australia

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the brushtail possum include a papillomavirus (Perrott et al., 2000) and a retrovirus (Baillie and Wilkins, 2001). Of these viruses, the adenovirus was selected as the most promising candidate for further investigation as a possible gene delivery vector.

Attempts to propagate the brushtail possum adenovirus were unsuccessful. Samples of possum intestinal contents that contained greater than 10^5 adenovirus particles per ml by EM were inoculated into a range of cell lines, including brushtail possum (*T. vulpecula*) kidney, potoroo (*Potorous tridactylus*) kidney, opossum (*Didelphis virginiana*) kidney, Vero, Madin–Darby bovine kidney, Norden laboratory feline kidney, Graham 293, primary brushtail possum (*T. vulpecula*) kidney and ovary, and primary chicken embryo fibroblasts. Embryonated chicken eggs were inoculated via the chorioallantoic membrane, the allantoic cavity, and yolk sac routes. Between three and seven passages were performed in each of these culture systems. There was no evidence of viral replication in any system. A molecular-based approach using degenerate PCR primers was, therefore, used to obtain DNA sequence information. In this paper, the sequence analysis of an adenovirus partially purified from the intestines of a brushtail possum is described. Adenoviral sequences obtained by PCR from intestinal contents of brushtail possums from different regions of New Zealand are also compared.

The prototype virus was partially purified from the large intestinal contents of a brushtail possum. Numerous adenovirus-like particles had been observed in this sample by EM (Rice and Wilks, 1996). One millilitre of intestinal contents were mixed with 8 ml phosphate buffered saline, pH 7.0 (PBS), and centrifuged for 20 min at $1000 \times g$. The supernatant was filtered through a 0.45 μm filter, layered onto a 25% sucrose cushion, and ultracentrifuged in a Beckman SW 40 Ti rotor at $125\,000 \times g$ for 1 h 45 min. Pellets were resuspended in 2 ml PBS and stored at −70 °C. Samples of intestinal contents from other brushtail possums, shown by EM to contain fewer adenovirus-like particles, were prepared using the Dynabeads® DNA Direct™ System 1 kit (Dynal). In this procedure the intestinal sample was first diluted 1:10 in resuspension buffer (500 mM Tris–HCl, 16 mM EDTA, 10 mM NaCl pH 9.0), vortexed, centrifuged at $21\,000 \times g$ for 10 min, and 50 μl of the supernatant used for DNA extraction. Further steps of the DNA purification were performed according to the manufacturer's instructions.

Highly degenerate oligonucleotide primers were designed from conserved regions of the conserved DNA polymerase (pol, an E2B gene) and hexon genes. Adenovirus sequences were obtained from peptide sequence databases (GenBank CDS translations, PDB, SwissProt, PIR) and nucleotide sequence databases (GenBank, EMBL, DDBJ, PDB). Seven DNA polymerase and 10 hexon sequences were aligned using CLUSTAL W (Thompson J.D. et al., 1994) with default parameters. The following primer pairs were used: DNA polymerase (AdVE2B F 5′-TCM AAY GCH YTV TAY GGB TCD TTT GC-3′, AdVE2B R 5′-CCA YTC HSW SAY RAA DGC BCK VGT CCA-3′); and hexon (AdVhexon F 5′-AAR GAY TGG TTY YTG RTN CAR ATG-3′, AdVhexon R 5′-CCV AGR TCN GTB ARD GYS CCC AT-3′). The primers correspond to the following ovine adenovirus isolate 287 nucleotides: E2B F, 5233-5208; E2B R, 4811-4837; hexon F, 16739-16762; and hexon R, 17142-17120. The primer pairs were expected to amplify fragments of approximately 450 and 400 bp, respectively. However, due to the unexpected amplification of the 3′-end of the penton base gene (protein III) and the following untranslated region with the hexon primers (in addition to the hexon product), an additional PoAdV-1-specific PCR could be performed to obtain the remainder of the penton base gene.

For the degenerate primer PCR, virus resuspended in PBS or viral DNA extracted by Dynabeads ® (1.0 μl) was added to the PCR reaction mix containing 10 mM Tris–HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 100 μM each dNTP, 0.5 U *Taq* DNA polymerase (Roche) and 1.0 μM of the degenerate primers in a final volume of 12.5 μl. Following an initial incubation at 94 °C for 1.5 min, PCR was performed for 30 cycles at 94 °C 5 s, 50 °C 5 s, and 72 °C 30 s. For the PoAdV-1 primer-specific long PCR, virus resus-

pended in PBS (1.0 μl) was added to the PCR reaction mix containing 20 mM Tris–HCl pH 8.0, 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 0.1% Triton X-100, 10 μg/ml nuclease-free BSA, 100 μM each dNTP, 0.5 U native *Pfu* DNA polymerase (Stratagene), and 0.2 μM PoAdV E2B F (5′-GTC TAT AAA AGG AAC GTC ATC AG-3′) and PoAdV penton R (5′-CTT TTC TGT TAC TTG TTT ACT CAC-3′) primers in a final volume of 12.5 μl. Following an initial incubation at 94 °C for 1.5 min, PCR was performed for 30 cycles at 94 °C 5 s, 60 °C 5 s, and 68 °C 4 min.

The PCR products were blunt-ended, phosphorylated, electrophoresed, excised, treated with agarase (Roche), and ligated to *Eco*RV digested, dephosphorylated pBluescript® II KS + (Stratagene), using the Rapid DNA ligation kit (Roche). *Escherichia coli* strain XL1-Blue MRF′ was transformed, and the colonies screened by PCR using vector primers T3 and T7. Sequencing grade DNA was prepared using the High Pure plasmid isolation kit (Roche). Both strands of the cloned degenerate PCR products were sequenced with T3 and T7 primers, and the 5′-end of the penton base gene by primer-walking, using the ABI Prism™ BigDye™ terminator cycle sequencing ready reaction kit (Perkin Elmer) and the ABI Prism™ 377 DNA sequencer (Perkin Elmer). Replicates of the sequences were aligned and edited using SEQUENCHER™ 3.1 (Gene Codes Corporation). Both ends of each sequence corresponding to the degenerate primers were removed. Sequences were translated in all six reading frames and compared with peptide sequence databases (GenBank CDS translations, PDB, SwissProt, PIR) using the homology search program BLAST 2.0.4 (Altschul et al., 1997), at either the Australian National Genomic Information Service (http://www.angis.org.au) or the dedicated adenovirus databank (GeneFarm, http://www.vmri.hu/blast.htm).

The putative penton base amino acid sequence was aligned to homologous adenoviral sequences by using the freely available program package BIOLOGY WORKBENCH 3.2 (http://workbench.sdsc.edu). The built-in Blast homology search was run against GenBank viral division sequences, which were then manually selected for the correct (non-repeated) sequences, followed by CLUSTAL W (Thompson J.D. et al, 1994) analysis. Phylogenetic calculations were performed using the PHYLIP program package version 3.6 (alpha) (Felsenstein, 1989) on manually edited forms of the alignment, shortened to the most conserved 436 residues-long part. PROTPARS was used for parsimony analysis, and PROTDIST (Dayhoff's PAM 001 matrix) followed by FITCH (global rearrangements) were used for distance matrix analysis. For bootstrap analysis, SEQBOOT (with 100 datasets) preceded the above calculations, after which CONSENSE was used to calculate the consensus tree. Phylogenetic relationships were visualised using TREEVIEW (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html) (Page, 1996) as described by Harrach and Benkö (1998). Applicable alignments, their edited format, and the calculated trees are available in Newick and graphical formats at http://www.vmri.hu/∼harrach. The nucleotide sequences reported in this paper have been deposited at GenBank, accession numbers AF249330–AF249333.

The degenerate DNA polymerase primers amplified a 425 bp fragment of the pol gene. The degenerate hexon primers amplified two fragments, a 416 bp fragment of the hexon gene, and, unexpectedly, a 578 bp genome fragment, covering from the 3′-end of the penton base gene to the 5′-end of the pVII gene (Fig. 1). The PoAdV-1 pol-penton primers amplified an approximately 8400 bp long genome fragment. Part of this clone was sequenced to obtain the 5′-end of the penton base gene. The putative penton base gene is 1344 bp in length, with a %G + C of 41.7, and encodes 447 amino acid residues. The highest Blast expectation values (Karlin and Altschul, 1990) were obtained with members of the proposed *Atadenovirus* genus, both for the complete penton base gene, and for the partial gene sequences (pol, hexon and pVII).

The high percentage of A + T in the sequenced part of the genome (58.3%) is in accordance with the earlier described characteristics of the atadenoviruses (Benkö and Harrach, 1998). The results of the detailed phylogenetic analysis (both parsimony and distance matrix analysis) confirmed the assumption that PoAdV-1 clusters with the pro-

posed atadenoviruses, represented in Fig. 2 by certain ruminant adenoviruses (BAdV-4 and OAV287), and an avian adenovirus (duck adenovirus 1, DAdV-1; syn. egg drop syndrome virus, EDS). Apart from the well separated clusters of the *Mastadenovirus*, *Aviadenovirus*, and *Atadenovirus genera*, a fourth cluster appeared, containing turkey adenovirus 3 (TAdV-3; syn. turkey hemorrhagic enteritis virus, THEV) and frog adenovirus 1 (FrAdV-1), members of another proposed genus (Davison et al., 2000) with the name *Siadenovirus* (indicating the occurrence of a sialidase gene) (Davison and Harrach, 2002).

In addition to the prototype sample from the South Island, a further six EM-positive samples were confirmed by PCR to contain PoAdV-1 (five from three locations on the North Island, and one from another South Island site). The partial pol, penton base/pVII, and hexon sequences proved to be identical from all seven samples.

Obtaining sequence information from a non-cultivable virus was made possible by the availability of numerous adenovirus sequences in public databases. Although no other marsupial adenovirus sequences were available, the homology at both the nucleotide and protein level for the pol and hexon genes enabled degenerate PCR primers to be designed. PCR using degenerate primers as a means to detect a non-cultivable putative adenovirus has been used previously, for guinea pig adenovirus (Pring-Åkerblom et al., 1997), and recently for red squirrel adenovirus (Sainsbury et al., 2001). However, only very short sequences from the hexon gene were obtained in both cases. The described degenerate pol primers have recently been used by Mária Benkö and her co-workers, to obtain the first DNA sequence data from fish and snake adenoviruses (personal communication).

At present, there are two recognised genera within the *Adenoviridae* family (Benkö et al., 2000), the *Mastadenovirus* and *Aviadenovirus* genera, but there is a proposal for a third genus, the *Atadenovirus* genus (Benkö and Harrach, 1998), which contains a number of recognised members (Boros et al., 1985; Benkö et al., 1988; Vrati et al., 1996; Harrach et al., 1997; Hess et al., 1997; Lehmkuhl and Cutlip, 1999; Russell and Benkö, 1999; Woods et al., 1999; Lehmkuhl et al., 2001). The results of the phylogenetic analysis strongly suggest that the brushtail possum adenovirus is a new member of this proposed genus. This preliminary classification is important for the design of further degenerate primers, as sequence alignments with members of the *Atadenovirus* genus will be more important than those with members of the other adenovirus genera. A definitive classification, however, may only be possible when

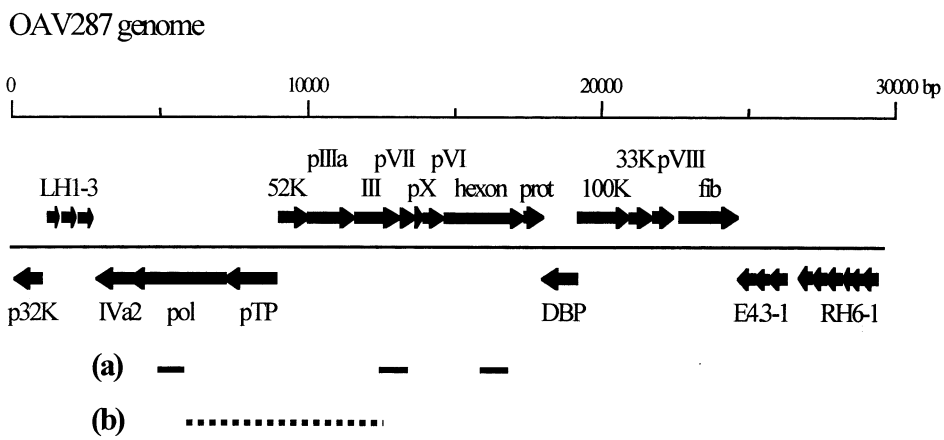

Fig. 1. Regions of the possum adenovirus genome that were amplified, cloned, and sequenced, compared with the genome of ovine adenovirus isolate 287, the proposed type species of the proposed genus *Atadenovirus*. (a) PCR products obtained with the degenerate primers (represented by solid lines). (b) The polymerase–penton base clone (represented by a dashed line), from which the 5′-end of the penton base gene was sequenced.

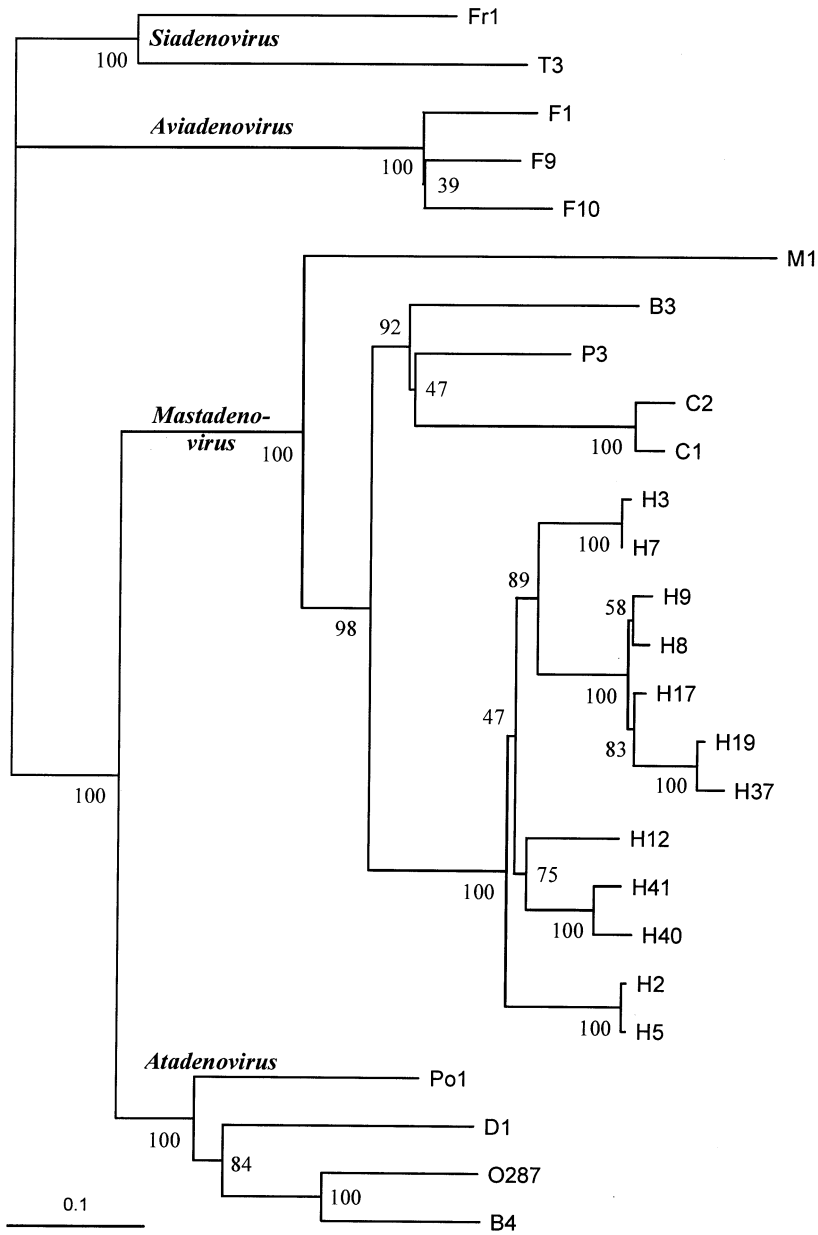

Fig. 2. Distance matrix analysis of penton base amino acid sequences. The final edited alignment had a length of 436 residues. The two established and two proposed genera are designated. Bootstrap values were calculated for 100 data sets and are shown. Virus types are represented by shortened forms of the ICTV abbreviations (Benkö et al., 2000) containing the host designation and type (or strain) number (B, bovine; C, canine; D, duck; F, fowl; Fr, frog; H, human; M, murine; O, ovine; P, porcine; Po, possum; T, turkey). Accessed data and their accession number in GenBank/EMBL database: B3, AF030154; B4, AF036092; C1, U55001; C2, U77082; D1, Y09598; F1, U46933; F9, AF083975; F10, M87008; Fr1, AF224336; H2, J01917; H3, Z29487; H5, M22141; H7, AD001675; H8, AJ249343; H9, AF217407; H12, X73487; H17, AF108105; H19, AF118438, H37, AF118437; H40, L19443; H41, AF105145; M1, U95438; O287, U40837; P3, AJ237815, Po, AF249332 (present paper); T3, AF074946.

complete gene sequences for the hexon and protease genes are available for analysis, along with the presence or absence of certain genus-specific genes. Examples of the latter would be the p32K gene, present only in atadenoviruses; the E1b regions, present in both atadeno- and mastadenoviruses; and the absence of proteins V, IX and the E3 region in atadenoviruses.

We have confirmed the presence of an adenovirus in brushtail possums. At present, only one genotype has been identified in brushtail possums from both the North and South Islands of New Zealand. A preliminary classification is that the possum adenovirus is a new member of the proposed genus *Atadenovirus*.

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