Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.
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

Received 8 June 2001; received in revised form 5 December 2001; accepted 5 December 2001

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. © 2002 Elsevier Science B.V. All rights reserved.

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 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
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 isolate 287 nucleotides: E2B F, 5233-5208; E2B R, 5052-5023; 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 MgCl₂, 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 (NH₄)₂SO₄, 2 mM MgCl₂, 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 AGC TGT TAC TTT TTT ACT CAC-3') and PoAdV penton R (5'-CTT TTC TGT TAC TTT ACT 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 EcoRV 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 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™ terminator cycle sequencing ready reaction kit (Perkin Elmer) and the ABI Prism™ 377 DNA sequencer (Perkin Elmer). Replicates of 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 (Benkő 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; 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.

Acknowledgements

Cell culture work was performed by Malcolm Rice and Laurie Sandall. Malcolm Rice and Matthew Perrott provided archival brushtail possum intestinal content samples and performed the EM analysis. Mária Benkő provided information and helpful discussions on the proposed Atadenovirus genus. Funding was provided by a grant from the New Zealand Ministry of Agriculture and Forestry. The Hungarian part of the work was supported by Hungarian Scientific Research Foundation Grant OTKA T030073.

References

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.

Baillie, G.J., Wilkins, R.J., 2001. Endogenous type D retrovirus in a marsupial, the common brushtail possum (Trichosurus vulpecula). J. Virol. 75, 2499–2507.

Benkő, M., Harrach, B., 1998. A proposal for a new (third) genus within the family Adenoviridae. Arch. Virol. 143, 829–837.

Benkő, M., Bartha, A., Wadell, G., 1988. DNA restriction enzyme analysis of bovine adenoviruses. Inter virology 29, 346–350.

Benkő, M., Harrach, B., Russell, W.C., 2000. Family Adenoviridae. In: Van Regenmortel, M.H.V., Fauquet, C.M., Bishop, D.H.L., Carstens, E.B., Estes, M.K., Lemon, S.M., Maniloff, J., Mayo, M.A., McGeoch, D.J., Pringle, C.R., Wickner, R.B., (Eds.), Virus Taxonomy. Seventh Report of the International Committee on Taxonomy of Viruses. Academic Press, San Diego, pp. 227–238.

Boros, G., Graf, Z., Benkő, M., Bartha, A., 1985. Isolation of a bovine adenovirus from fallow deer (Dama dama). Acta Vet. Hung. 33, 119–123.

Cowan, P.E., 1996. Possum biocontrol: prospects for fertility regulation. Reprod. Fertil. Dev. 8, 655–660.

Davison, A., Harrach, B., 2002. Genus Stadenovirus. In: Timona, C.A., Darai, G., (Eds.), The Springer Index of Viruses, Springer, Heidelberg, in press.

Davison, A.J., Wright, K.M., Harrach, B., 2000. DNA sequence of frog adenovirus. J. Gen. Virol. 81, 2431–2439.

Felsenstein, J., 1989. PHYLIP—phylogeny inference package (version 3.2). Cladistics 5, 164–166.

Harrach, B., Benkő, M., 1998. Phylogenetic analysis of adenovirus sequences. Proof of the necessity of establishing a third genus in the Adenoviridae family. In: Wold, W.S.M. (Ed.), Adenovirus Methods and Protocols. Humana Press, Totowa, NJ, USA, pp. 309–339.

Harrach, B., Mecham, B.M., Benkő, M., Adair, B.M., Todd, D., 1997. Close phylogenetic relationship between egg drop syndrome virus, bovine adenovirus serotype 7, and ovine adenovirus strain 287. Virology 229, 302–306.

Hess, M., Blocker, H., Brandt, P., 1997. The complete nucleotide sequence of the egg drop syndrome virus—an intermediate between mastadenoviruses and avianadenoviruses. Virology 238, 145–156.

Karlin, S., Altschul, S.F., 1990. Methods for assessing the statistical significance of molecular sequence features by using general scoring schemes. Proc. Natl. Acad. Sci. USA 87, 2264–2268.

Lehmkuhl, H.D., Cutlip, R.C., 1999. A new goat adenovirus isolate proposed as the prototype strain for goat adenovirus serotype 1. Arch. Virol. 144, 1611–1618.

Lehmkuhl, H.D., Hobbs, L.A., Woods, L.W., 2001. Characterization of a new adenovirus isolated from black-tailed deer in California. Arch. Virol. 146, 1187–1196.

Page, R.D.M., 1996. TREEVIEW—an application to display phylogenetic trees on personal computers. Comput. Appl. Biosci. 12, 357–358.

Perrott, M.R.F., Meers, J., Greening, G.E., Farmer, S.E., Lugton, I.W., Wilks, C.R., 2000. A new papillomavirus of possums (Trichosurus vulpecula) associated with typical wart-like papillomas. Arch. Virol. 145, 1247–1255.

Pracy, L.T., 1974. Introduction and liberation of the opossum (Trichosurus vulpecula) into New Zealand. New Zealand Forest Service Information Series No. 45.

Pring-Akerblom, P., Blazek, K., Schramlova, J., Kunstyr, I., 1997. Polymerase chain reaction for detection of guinea pig adenovirus. J. Vet. Diagn. Invest. 9, 232–236.

Rice, M., Wilks, C.R., 1996. Virus and virus-like particles observed in the intestinal contents of the possum, Trichosurus vulpecula. Arch. Virol. 141, 945–950.

Russell, W.C., Benkő, M., 1999. Adenoviruses (Adenoviridae): animal viruses. In: Webster, R.G., Granoff, A. (Eds.),
Encyclopedia of Virology. Academic Press, London, pp. 14–21.

Sainsbury, A. W., Adair, B., Graham, D., Gurnell, J., Cunningham, A. A., Benkő, M., Papp, T., 2001. Isolation of a novel adenovirus associated with splenitis, diarrhoea, and mortality in translocated red squirrels, Sciurus vulgaris. Erkrankungen der Zootiere 40, 265–270.

Sutherland, O. R. W., Cowan, P. E., Orwin, J., 1996. Biological control of possums Trichosurus vulpecula and rabbits Oryctolagus cuniculus in New Zealand. Wildlife Biol. 2, 165–170.

Thompson, J. D., Higgins, D. G., Gibson, T. J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

Vrati, S., Brookes, D. E., Strike, P., Khatari, A., Boyle, D. B., Both, G. W., 1996. Unique genome arrangement of an ovine adenovirus-identification of new proteins and proteinase cleavage sites. Virology 220, 186–199.

Woods, L. W., Hanley, R. S., Chiu, P. H., Lehmkuhl, H. D., Nordhausen, R. W., Stillian, M. H., Swift, P. K., 1999. Lesions and transmission of experimental adenovirus hemorrhagic disease in black-tailed deer fawns. Vet. Pathol. 36, 100–110.