

## Characterisation of two enteroviruses isolated from Australian brushtail possums (*Trichosurus vulpecula*) in New Zealand

### Brief Report

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**Summary.** Two enteroviruses, designated W1 and W6, were isolated from intestinal contents of Australian brushtail possums (*Trichosurus vulpecula*) in New Zealand. The genomic sequences of W1 and W6 were 7390 and 7391 nucleotides (nt), respectively. Genetically, possum isolates W1 and W6 were related to bovine enterovirus serotype 2 (BEV-2) strains, especially to the strain PS87/Belfast, based on the capsid protein sequence. However, W1 and W6 formed a clade that was distinct from PS87/Belfast based on nucleotide sequences of the 3' and 5'-non-translated region and in the amino acid sequences of 2A, 3C and 3D. Possum isolates W1 and W6 grew more readily in possum kidney cells than in Madin-Darby bovine kidney (MDBK) cells, suggesting that co-evolution of W1 and W6 with possums has made them more adapted to possum cells.

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Australian brushtail possums (*Trichosurus vulpecula*) were introduced into New Zealand from Australia in the early 19th century to establish a fur industry [12]. They have since become the most important vertebrate pest in New Zealand [3]. While conventional control methods such as poisoning and trapping are effective, the risks posed by the reliance on poisons (environmental, trade, public acceptance and cost) threaten their continued long-term use and sustainability. Biological management was identified as a research priority by the National Science Strategy Committee for possum control in New Zealand. Viruses can be used as pathogens or transmissible vectors to deliver bioactive molecules for possum management. Considerable progress has been made in the past 10 years in research on possum

Nucleotide sequence data reported here are available in the GenBank database under the accession numbers of AY462106 and AY462107 for possum isolates W1 and W6, respectively.

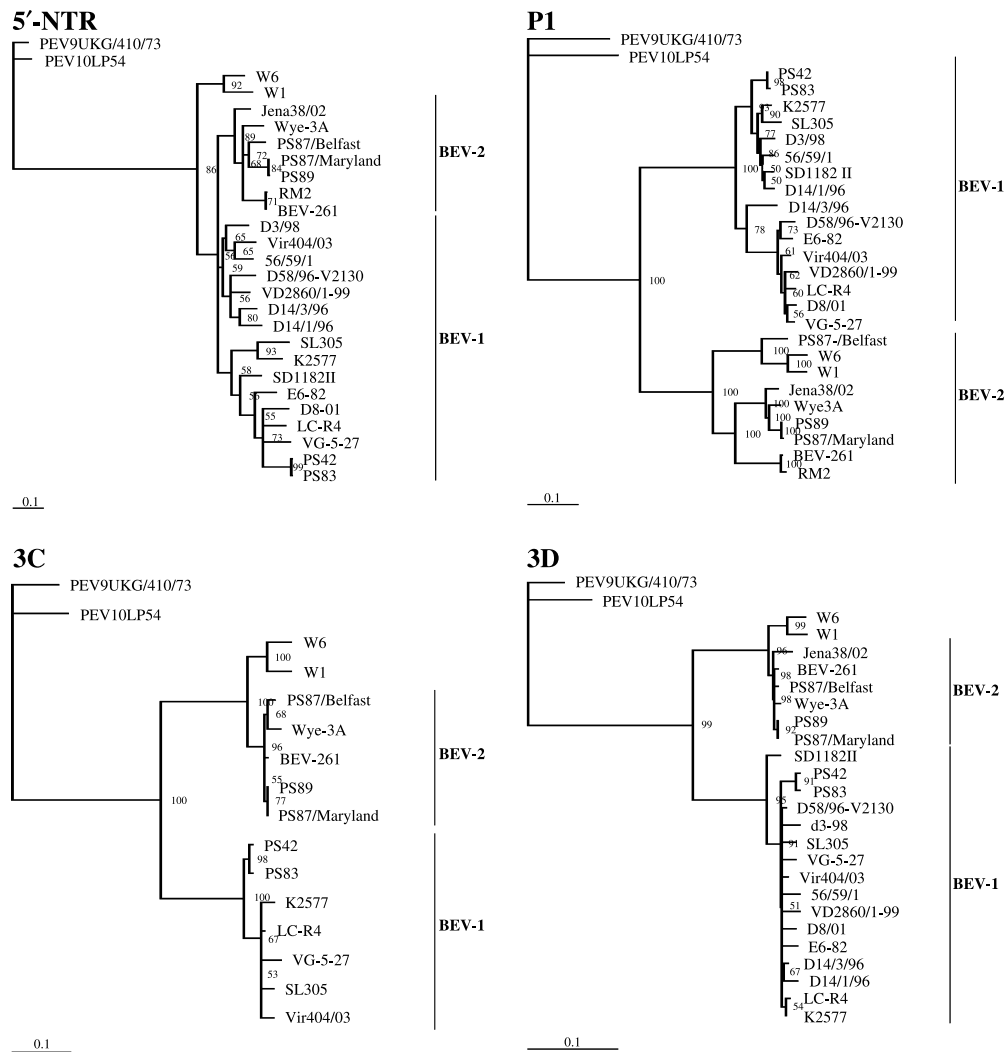
viruses for possum biological management. Herpesvirus, adenovirus, coronavirus, and coronavirus-like virus were identified by electron microscopy from possum intestinal contents [14] and a papillomavirus was identified from a papillomatous lesion on the tail of a possum [11]. An endogenous type D retrovirus was also detected in possums [1]. These results suggested that possums, like other mammals, harbour various virus species and some of them could be developed as virus vectors. However, culture of these viruses in cell culture systems was unsuccessful [20]. In this paper we report the culture, isolation and characterisation of two enteroviruses from possums in New Zealand.

Possums were shot from the Shannon area of the lower North Island, New Zealand. A total of 23 possums were shot from trees near sheep farmland and an adjacent pine forest in June 2002. Possums were checked, weighed and sexed. They appeared healthy with normal fat reserves. They weighed from 1.4 to 3.6 kg with a mean of 2.6 kg and comprised of 12 males and 11 females. Fur around the abdomen was wetted with a disinfectant, 1% VirKon (Antec International Limited, Suffolk, UK), prior to necropsy. Intestinal samples were collected from the colon in the area anterior to faecal pellet formation and stored at  $-75^{\circ}\text{C}$ . A 20% (w/v) suspension of each sample was made in virus transport media (Eagle's MEM (Gibco<sup>TM</sup>, Invitrogen Corporation, Carlsbad, California, USA) containing 0.5% bovine serum albumin, 0.03% gentamycin, 0.01% kanamycin, 0.01% streptomycin and 0.06% penicillin) for virus isolation. Cell cultures used for virus isolation were opossum (*Didelphis virginiana*) kidney (OK) cells (ATCC CRL-1840), potoroo (*Potorous tridactylis*) kidney cells (Pt K1) (ATCC CCL-35), African green monkey (*Cercopithecus aethiops*) kidney cells (Vero) (ATCC CCL-81) and a primary possum (*Trichosurus vulpecula*) kidney cell culture (PPK). The PPK cell culture was established in this laboratory. Cytopathic effect (CPE) was observed in PPK cells, which were inoculated with filtrates of suspensions from two pooled possum intestinal content extracts. CPE was not observed in the OK, Pt K1 and Vero cells, which were similarly inoculated. The affected cells of the PPK culture became rounded up and swollen, then quickly detached. Filtration of the agent through a 0.22- $\mu\text{m}$  filter did not prevent the development of the CPE. The two possum viral isolates, designated as W1 and W6, were further purified twice by plaque purification. The buoyant density of the isolates in CsCl was 1.33 g/ml, a mean from five independent measurements. The isolates were stable at pH 3.0, and 5'-bromodeoxyuridine (100  $\mu\text{g/ml}$ ) did not inhibit the growth of W1 and W6 isolates. Under the electron microscope, the negative-stained virus isolates were small, non-enveloped, icosahedral symmetrical particles, 27–30 nm in diameter. Empty capsids were frequently observed (data not shown).

The cell susceptibility to W1 and W6 was further tested on PPK, Vero, OK, Pt K1, MDBK (ATCC CCL-22) and Hep-2 (ATCC CCL-23) cells by the production of CPE in which infected cells rounded up, showed shrinkage and nuclear pyknosis, and subsequently detached from the surface of the culture plate. Briefly, confluent MDBK, Hep-2, PPK, OK, Pt K1 and Vero cell monolayers in 6-well tissue culture plates (Nunc<sup>TM</sup>) were washed three times with phosphate buffered saline (PBS<sup>-</sup>, Ca<sup>2+</sup> and Mg<sup>2+</sup> free). For each 6-well plate of a cell type, two wells

were inoculated with W1 isolate (0.5 ml/well of MEM containing  $10^3$  TCID<sub>50</sub> W1), two wells were similarly inoculated with W6 isolate, and the remaining two wells had 0.5 ml/well of MEM only added and served as cell controls. The W1 and W6 isolate stock had been passaged in PPK cells three times prior to this assay. Following incubation of the plates for 90 min at 37 °C in a humidified 5% CO<sub>2</sub> incubator, the viral medium was removed and the cell monolayers were washed three times with PBS<sup>-</sup>, and 3 ml/well of culture medium (Eagle's MEM supplemented with antibiotics and 5% foetal bovine serum) was added. The plates were then incubated at 37 °C in a humidified 5% CO<sub>2</sub> incubator, and the development of CPE was observed under a microscope. While typical CPE was not observed when W1 and W6 were cultured on OK and Hep-2 cells, a slow development of CPE was observed on Pt K1 cells, where only a few isolated CPE foci were evident 6 days post-inoculation (p.i.). The development of CPE to approximate 50% of cells on the cultures of PPK, MDBK and Vero cells took approximately 36, 36 and 72 h p.i., respectively, for W1 isolate, and approximately 36, 72 and 72 h p.i. for W6 isolate. W1 isolate produced complete CPE in PPK, MDBK and Vero cells at approximately 48, 48 and 96 h p.i., respectively. W6 isolate produced complete CPE in PPK cells at 48 h p.i., while CPE was incomplete in MDBK and Vero cells after the 6-day observation period following the inoculation.

W1 and W6 were suspected to be enteroviruses based on their biophysical characteristics. A reverse transcriptase-polymerase chain reaction (RT-PCR) targeting on the 5'-non-translated region (5'-NTR) of enteroviruses was conducted under conditions described by Zoll et al. [22]. Briefly, Primer 3 (5'-ATTGT CACCATAAGCAGCCA-3') was used for cDNA synthesis using Expand<sup>TM</sup> Reverse Transcriptase (Boehringer Mannheim GmbH, Mannheim, Germany); Primer 1 (5'-CAAGCACTTCTGTTTCCCCGG-3') and Primer 3 were used for PCR using Taq DNA polymerase (Life Technologies, Rockville, USA). Amplification was carried out in a thermocycler (Progene, Techne, Cambridge, UK) with a cycle program of 94 °C for 30 sec, 45 °C for 45 sec, and 72 °C for 60 sec. The reaction started at 94 °C for 2 min, followed by 35 repeats of the cycle program with the final extension at 72 °C for 7 min. An approximately 572-bp fragment was amplified by the RT-PCR from RNA samples extracted from W1- and W6-infected PPK cells (QIAamp Viral RNA Mini Kit). The amplified fragments were cloned in the pGMT<sup>®</sup>-T Easy vector (Promega Corporation, Madison, WI, USA) and sequenced at the Waikato DNA Sequencing Facility (University of Waikato, Hamilton, New Zealand). The 5' and 3' ends of the viral RNA were subsequently amplified using a 5'/3' RACE Kit (Roche Diagnostic GmbH, Mannheim, Germany). The remaining viral genomic sequences were further amplified using primers specific to W1 or W6 isolate by the Expand High Fidelity PCR System (Roche Diagnostic GmbH), and subsequently cloned into pCR<sup>®</sup>II-TOPO<sup>®</sup> (Invitrogen Corporation) and sequenced with both sense and anti-sense primers. Viral sequences were analysed using the Wisconsin Package<sup>TM</sup> Version 10.3 (Accelry Inc., San Diego, CA, USA). GAP was used for pairwise comparison between viral sequences. The optimal and suboptimal secondary structures for the 5'-NTR and 3'-NTR were computed using the energy minimisation



**Fig. 1.** Phylogenetic trees of the 5'-NTR nucleotide sequence and P1, 3C and 3D amino acid sequences of W1 and W6 possum isolates and strains of BEV in the database. Details of the software packages and algorithms used are given in the text. The BEV strains and their sequence accession numbers used in the analysis were as follows: 56/59/1 (DQ092778, DQ092779), BEV-261 (DQ092770), D 14/1/96 (DQ092780, DQ092781), D 14/3/96 (DQ092786, DQ092787), D 3/98 (DQ092790, DQ092791), D 58/96-V2130 (DQ092772, DQ092773), D 8/01 (DQ092782, DQ092783), E 6-82 (DQ092776, DQ092777), Jena 38/02 (DQ092788, DQ092789), K 2577 (AF123432), LC-R4 (DQ092769), PS 42 (DQ092792), PS 83 (DQ092793), PS 87/Belfast (DQ092794), PS 87/Maryland (AY508696), PS 89, DQ092795, RM2 (X79369), SD 1182 II (DQ092784, DQ092785), SL 305 (AF123433), VD 2860/1-99 (DQ092774, DQ092775), VG-5-27 (D00214) and Vir 404/03 (DQ092771). Two porcine enteroviruses PEV9/UK G410/73 (AF363453) and PEV10/LP54 (AF363455) were used as outgroups. Branch lengths are proportional to the genetic divergence. The scale bar represents 0.1 substitutions per nucleotide site. Numbers at nodes represent percentages of bipartitions in intermediate trees that have been generated in 25000 puzzling steps

method by the MFOLD (Version 2.3) [23]. Multiple sequences were aligned by the CLUSTAL X programme (Version 1.8) [19]. Phylogenetic relationships were analysed by the TREE-PUZZLE programme (Version 5.2) [18]. Neighbouring-joining trees were constructed by the quartet puzzling method [16, 17].

Recombinant plasmids with W1- or W6-derived cDNA inserts were sequenced to compile and determine the nucleotide sequence of the isolates. The genomic sequences of W1 and W6 were 7390 and 7391 nt, respectively, excluding the poly (A) tract. The genomic organisations of W1 and W6 were proposed following the comparison of their nucleotide sequences with those of other enteroviruses. The 5'-NTR regions of W1 and W6 were 818 and 819 nt, respectively, followed by a single long ORF of 6498 nt, and a 3'-NTR of 74 nt, including the stop codon, and tailed by approximate 65 nt of poly (A) tract. The 5'-NTR of W1 isolate shared 85.3% identity with that of W6 isolate. Possum isolates W1 and W6 shared approximately 71.0–80.8% with that of BEV isolates. Like other bovine enteroviruses, possum isolates W1 and W6 had an approximate 100 additional nucleotides 'insertion' at the 5' end of the 5'-NTR. The phylogenetic relationships of possum isolates and BEV isolates were analysed based on the nucleotide sequence of 5'-NTR using porcine enteroviruses as outgroups, W1 and W6 formed a distinct clade segregated from all BEVs that descended from a separate common ancestral node (Fig. 1). There were three clusters descended from the BEV clade. All 7 BEV-2 strains clustered together, while BEV-1 strains clustered in two groups. In the inferred tree of the 3'-NTR of 14 strains, W1 and W6 formed a separate branch that joined a common ancestral node with that of BEV-2 (data not shown). The overall secondary structures of the 5'-NTR and 3'-NTR of W1 and W6 were similar to that of BEVs (data not shown).

There are differences between BEV-1 and BEV-2 isolates in the dipeptide pairs of the polyprotein cleavage junctions in 1C-1D (Q-N vs. Q-G), 1D-2A (L(Y)-G vs. T(A)-G) and 2B-2C (Q-S vs. Q-A). The polyprotein cleavage junctions of W1 and W6 were consistent with those of BEV-2 isolates. The deduced capsid polyprotein, P1, of W1 and W6 consisted of 832 amino acid residues. W1 and W6 shared 93.2% identity in the P1 amino acid sequence, and possum isolates shared approximately 78 and 88% identity with BEV-2 strains PS 89 and PS87/Belfast, respectively. Phylogenetic analysis of the P1 amino acid sequences of W1 and W6 with 22 bovine isolates showed that W1 and W6 clustered with PS 87/Belfast within the BEV-2 clade (Fig. 1). The close phylogenetic relationship between W1, W6 and PS 87/Belfast was also evident when the amino acid sequences of the 1B (VP2), 1C (VP3) and 1D (VP1) regions were analysed individually (data not shown). In comparison to strains of BEV-1, W1 and W6 had two amino acid residues missing at the C-terminal of 1B (VP2) and five amino acid residues missing at the N-terminal of 1D (VP1), a common feature in BEV-2 strains.

In the non-structural protein region, the catalytic triads of 2A (His-20, Asp-38 and Cys-109) and 3C (His-40, Glu-71 and Cys-147) were conserved in possum isolates W1 and W6. Similar to the phylogenetic trees of the structural proteins, W1 and W6 were more closely associated to each other than they were with other BEV-2 strains in the non-structural proteins. W1 and W6 formed a distinct

clade segregated from other BEV-2 strains, including PS 87/Belfast in the 3C and 3D regions (Fig. 1). Consistent phylogenetic trees were obtained by both JTT and WAG evolution models on the amino acid sequences of structural and non-structural proteins, and by TN and HKY evolution models on the nucleotide sequences of 5'-NTR and 3'-NTR (data not shown).

Bovine enteroviruses are classified into two serotypes within the species of *Bovine enterovirus* of the genus *Enterovirus* [7]. Genetically, possum isolates W1 and W6 were closely related to BEV-2 strain PS 87/Belfast based on the capsid proteins. PS 87 was isolated in cell culture from faeces of a 2-week-old calf with oedema of spinal cord in Pennsylvania State University in 1962 [4]. There are two full genomic sequences (AY508696 and DQ092794) and one partial sequence (X79368) for strain PS 87 in the public domain [6, 10, 21]. The sequence of PS 87/Belfast DQ092794 is identical to the PS 87 partial sequence (X79368), while PS 87/Maryland (AY508696) and PS 89 share 99.8% identity in the P1 protein sequence and was suggested as probable cross contaminations of the viral stocks [21]. Knowles and Barnett designated five subtypes in BEV-2 [8]. Strain BEV-261 was assigned in subtype 1 of BEV-2, and it did not cross-react with PS 87/Pirbright and PS 89/Pirbright in virus cross-neutralisation assays [8], even though BEV-261 and PS 89 shared 85.6% identity in their P1 amino acid sequence. It remains to be determined if W1, W6 and PS 87/Belfast belonging to the same subtype, as they shared approximately 88% identity in P1. Serological comparison between possum isolates W1 and W6 and other BEVs will help to further differentiate these viruses.

Although possum isolates W1 and W6 were closely related to PS 87/Belfast based on capsid proteins, possum isolates exhibited distinct evolutionary pathways from PS87/Belfast based in that W1 and W6 formed a distinct clade from PS87/Belfast in nucleotide sequences of 5'-NTR and 3'-NTR and in amino acid sequences of non-structural proteins 2A, 3C and 3D. The 5'-NTR, 3'-NTR and 3D are involved in viral genome replication and translation, while 2A, 3C and 3CD are involved in the posttranslational cleavage of viral polyprotein (for review: [13]). The phylogenic differences in 3'-NTR, 5'-NTR and in 2A, 3C and 3D between possum isolates W1 and W6 and strains of BEV-2 may reflect the characteristics of co-evolution of these viruses with different host systems. In this study, PPK, MDBK and Vero were permissive cells for W1 and W6, but possum isolates appeared to grow more readily in possum cells (PPK cells) than in MDBK and Vero cells, especially for the W6 isolate. The co-evolution of W1 and W6 isolates and possums would have made them more adapted to possum cells.

BEVs were isolated from bovine faecal samples previously in Australia [15]. The representative serological prototype strains of BEV, isolated from Queensland, Australia, were all genetically BEV-1 viruses [9]. BEVs were also isolated from cattle in New Zealand [2, 5], but their genomic characteristics remain to be determined. More genomic data from animal enteroviruses isolated in New Zealand are required to establish their relationships with W1 and W6 isolates. The sequences of enteroviruses in the public domain, including recently published sequences of six American and eleven German BEV isolates by Zell et al. [21],

have significantly accelerated the characterisation process for W1 and W6 isolates. The relationships of W1 and W6 with other animal enteroviruses will be more completely defined when more sequences of animal enteroviruses become available.

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