Virus Research 242 (2017) 106–121

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Complete genomic characterisation of two novel poxviruses (WKPV and EKPV) from western and eastern grey kangaroos

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ARTICLE INFO

Keywords:
Macropus
Kangaroo
Macropod
Poxvirus
Thylacopoxvirus
Cullin

ABSTRACT

Poxviruses have previously been detected in macropods with cutaneous papillomatous lesions, however to date, no comprehensive analysis of a poxvirus from kangaroos has been performed. Here we report the genome sequences of a western grey kangaroo poxvirus (WKPV) and an eastern grey kangaroo poxvirus (EKPV), named for the host species from which they were isolated, western grey (*Macropus fuliginosus*) and eastern grey (*Macropus giganteus*) kangaroos. Poxvirus DNA from WKPV and EKPV was isolated and entire coding genome regions determined through Roche GS Junior and Illumina Miseq sequencing, respectively. Viral genomes were assembled using MIRA and SPAdes, and annotations performed using tools available from the Viral Bioinformatics Resource Centre. Histopathology and transmission electron microscopy analysis was also performed on WKPV and its associated lesions.

The WKPV and EKPV genomes show 96% identity (nucleotide) to each other and phylogenetic analysis places them on a distinct branch between the established *Molluscipoxvirus* and *Avipoxvirus* genera. WKPV and EKPV are 170 kbp and 167 kbp long, containing 165 and 162 putative genes, respectively. Together, their genomes encode up to 47 novel unique hypothetical proteins, and possess virulence proteins including a major histocompatibility complex class II inhibitor, a semaphorin-like protein, a serpin, a 3-β-hydroxysteroid dehydrogenase/δ 5 → 4 isomerase, and a CD200-like protein. These viruses also encode a large putative protein (WKPV-WA-039 and EKPV-SC-038) with a C-terminal domain that is structurally similar to the C-terminal domain of a cullin, suggestive of a role in the control of host ubiquitination. The relationship of these viruses to members of the *Molluscipoxvirus* and *Avipoxvirus* genera is discussed in terms of sequence similarity, gene content and nucleotide composition. A novel genus within subfamily *Chordopoxvirinae* is proposed to accommodate these two poxvirus species from kangaroos; we suggest the name, Thylacopoxvirus (thylaco-: [Gr.] *thylakos* meaning sac or pouch).

1. Introduction

1.1. Macropodid and other hosts

Western grey kangaroos (*Macropus fuliginosus*) and eastern grey kangaroos (*Macropus giganteus*) are Australian macropodid marsupials classified within the 'least concern' category of the IUCN red list of threatened species (Burbidge et al., 2016a, 2016b). *M. fuliginosus* inhabits a wide swathe of southern Australia, from Western Australian coastal regions to south-western Queensland, western New South Wales

Abbreviations: APC, anaphase-promoting complex; APV, avipoxvirus; ATIp, A-type inclusion protein; ChPV, chordopoxvirus; CRL, cullin-RING E3 ligase; CRV, crocodile poxvirus; CTD, C-terminal domain; EEV, enveloped extracellular virus; EKPV, Eastern grey kangaroo poxvirus; GATU, Genome Annotation Transfer Utility; IMV, intracellular mature virus; ITR, inverted terminal repeat; IUCN, International Union for Conservation of Nature; KPV, kangaroo poxvirus; MOCV, *Molluscum contagiosum virus*; MYXV, *Myxoma virus*; ORF, open reading frame; ORFV, *Orf virus*; PACR, poxvirus APC/cyclosome regulator; RMSD, root mean squared deviation; SQPV, squirrel poxvirus; TKPV, *Turkeypox virus*; VARV, *Variola virus*; VOCs, Viral Orthologous Clusters; WKPV, Western grey kangaroo poxvirus

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http://dx.doi.org/10.1016/j.virusres.2017.09.016
Received 7 August 2017; Received in revised form 23 September 2017; Accepted 25 September 2017
Available online 27 September 2017
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and Victoria. *M. giganteus* is endemic to eastern mainland Australia (Queensland, New South Wales, Victoria, and eastern South Australia), and a subspecies, *M. g. tasmaniensis* is found in eastern Tasmania (Kirsch and Poole, 1972). *M. fuliginosus* and *M. giganteus* are thought to have diverged from a common macropodid ancestor during the Pliocene, approximately 2.5 ± 1.5 million years ago (Meredith et al., 2009).

Cutaneous poxvirus infections of *M. fuliginosus* and *M. giganteus* have been previously reported in the scientific literature (McKenzie et al., 1979; Rothwell et al., 1984). Clinically, solitary or multiple wart-like, raised cutaneous lesions are seen on the extremities, especially the face, feet and tail. These lesions are thought to spontaneously regress after approximately three months, leaving a hairless scar. A range of other macropodid marsupials can also manifest clinically similar lesions, including *Macropus rufus*, *Macropus robustus*, *Macropus eugenii*, *Macropus agilis*, *Wallabia bicolor*, and *Setonix brachyurus* (Ladds, 2009). Poxviral diseases in Australian animals have also been reported in crocodiles, possums and more recently a flying fox (Buenviaje et al., 1992; Vogelnest et al., 2012; O'Dea et al., 2016). Prior to this study, of the poxviruses found in Australian mammals, only pteropox virus has had the entire coding region of its genome sequenced.

1.2. Poxviruses

Members of the virus family *Poxviridae* have ovoid to brick-shaped, relatively large, enveloped virions that contain a single, linear molecule of covalently closed double-stranded DNA, 130–375 kbp long. The family is divided into two subfamilies, *Entomopoxvirinae*, whose members infect insects, and *Chordopoxvirinae*, whose members infect vertebrates (Adams et al., 2017). The latter are currently divided into 12 genera, but several viruses, including the ones reported here, have been proposed to require the designation of new genera (Adams et al., 2017). Poxviruses that infect humans include Variola virus (VARV), the agent of smallpox that has been eradicated as a human disease, and Molluscum contagiosum virus (MOCV), which produces wart-like lesions. Although the division of viruses between the various genera groups the poxviruses by host range to some extent, VARV and MOCV are in separate genera and are considered very different viruses. Thus, given only genomic sequence data it is likely impossible to predict the host range of a particular poxvirus.

1.3. Poxvirus infections of macropods

Despite poxvirus-associated lesions being relatively common in macropods, there are few reports on the histopathological features of the disease, and no complete characterisations of the associated viruses. In this study, we describe the full genomes of two novel poxvirus species extracted from lesional material from two kangaroo species. We assigned names and strains for western grey (WKPV-WA) and eastern grey (EKPV-SC) kangaroo poxviruses, based on their respective hosts from Western Australia (WA) and the Sunshine Coast (SC). There have not been any documented reports of human poxvirus lesions following contact with macropod skin lesions, and currently there is no suspicion that these poxviruses infect humans or other animals. However, the enormous geographic range inhabited by these animals would suggest that they at least come into contact with a large assortment of other animals and are therefore of interest as potential agents of cross-species infections (Parrish et al., 2008).

2. Methods

2.1. Sample collection, extraction and processing

The western grey kangaroo joey was a roadkill specimen found in the vicinity of Greenmount State Forest near Perth, Western Australia, in May 2013. The carcase was taken to a nearby veterinary hospital where it was stored frozen (−20 °C), until being used in a wildlife necropsy practical class for the Murdoch University Wildlife Association. Representative samples were collected from the skin mass on the dorsal muzzle.

An orphaned 9 kg, juvenile, female eastern grey kangaroo was found on the campus of the University of the Sunshine Coast, Sippy Downs, Queensland, in April 2010. The kangaroo was euthanised due to severe wounds likely caused by a dog attack, and a fresh frozen sample of papillomatous skin was sent to Murdoch University for sequencing purposes.

For the western grey kangaroo sample, lesional tissue was fixed in 10% neutral buffered formalin, and a representative subsample was taken from the skin mass for routine histopathology. Processed tissue (Leica EG 1150C automatic processor, Leica Microsystems) was embedded in paraffin wax, blocks were sectioned at 5 µm with a Leica 2135 microtome (Leica Microsystems) and placed on silanised glass microscope slides (ProSciTech), then stained with haematoxylin and eosin and permanently mounted with DPX. Slides were examined using an Olympus BX-50 light microscope.

For transmission electron microscopy, formalin fixed tissue was finely minced and a drop of this material was placed onto a 0.35% formvar-coated grid and allowed to settle for 5 min at room temperature. The excess liquid was removed using filter paper and the dried grid was placed onto a drop of 1% phosphotungstic acid (w/v) in 0.01 mol/L tris buffer pH 7.3 for 30 s. Excess liquid was again removed with filter paper and the dried grid loaded into a BioTwin CM 100 transmission electron microscope (Philips, Eindhoven, Holland) for visualization of negatively stained virus particles at an accelerating voltage of 80 kV.

An unfixed sample of lesional skin was finely minced using sterile scalpel blades and total DNA was extracted using a DNeasy tissue kit (Qiagen) according to the manufacturer's instructions. The DNA library was prepared using the Roche Rapid Library kit according to the Rapid Library Preparation Method Manual. Sequencing was carried out on a GS Junior sequencer using GSJR titanium sequencing kit and GSJR Pico titre plate according to the Roche Sequencing Method Manual.

For the eastern grey kangaroo skin lesion, DNA was extracted using a Qiagen DNEasy Blood and Tissue kit (Qiagen) according to the manufacturer's instructions. Library preparation was undertaken using a Nextera XT DNA Library Prep Kit (Illumina), according to the manufacturer's instructions, with the following minor modifications. During the NTA reaction, the cycling step of 55 °C for 5 min was increased to 7 min and for the PCR bead clean-up, 30 µL of AMPure beads was used. The library was sequenced on an Illumina MiSeq V2 2 × 300 flowcell using standard Illumina MiSeq protocols.

In order to define the number of repeats in a region which could not be clearly defined using short read sequencing, the following primers were developed EGK poxrpt 902 F (AAAACGCAGCGCACGGAC) and EGK pox rpt 1093 R (CGCGCATGCCCCGTATCT). PCR reactions were performed with AmpliTaq Gold 360 mastermix (Life Technologies) and primers at a final concentration of 250 nmol/L. Bands of the expected size were excised from a 2% agarose gel, purified using a Wizard Gel and PCR purification kit (Promega), and Sanger sequencing reactions performed by the Australian Genome Research Facility.

2.2. Assembly protocol

Sequence data for kangaroo poxvirus (KPV) species WKPV and EKPV were obtained in 2013 and 2017, respectively. Roche 454 sequence data were obtained for WKPV species and assembled using MIRA assembler (Chevreux et al., 2004) under the "genome, denovo, accurate" parameters. Pair-end Illumina sequence data were obtained for EKPV. Different assembly trials used reads filtered against *Macropus eugenii* genomic sequence (INSDC: ABQO000000000.2; the closest available marsupial sequence to *M. giganteus*), and/or human and bacterial sequences, that were additionally followed by duplicate read removal using FastUniq (Xu et al., 2012) and assembled using SPAdes (Bankevich et al., 2012) under the "careful" parameter. Preliminary

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