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A single bat species in Cameroon harbors multiple highly divergent papillomaviruses in stool identified by metagenomics analysis

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ABSTRACT

A number of PVs have been described in bats but to the best of our knowledge not from feces. Using a previously described NetoVIR protocol, *Eidolon helvum* pooled fecal samples (Eh) were treated and sequenced by Illumina next generation sequencing technology. Two complete genomes of novel PVs (EhPV2 and EhPV3) and 3 partial sequences (BATPV61, BATPV890a and BATPV890b) were obtained and analysis showed that the EhPV2 and EhPV3 major capsid proteins cluster with and share 60–64% nucleotide identity with that of *Rousettus aegyptiacus* PV1, thus representing new species of PVs within the genus *Psipapillomavirus*. The other PVs clustered in different branches of our phylogenetic tree and may potentially represent novel species and/or genera. This points to the vast diversity of PVs in bats and in *Eidolon helvum* bats in particular, therefore adding support to the current concept that PV evolution is more complex than merely strict PV-host co-evolution.

1. Introduction

The family *Papillomaviridae* comprises a large family of non-enveloped icosahedral viruses with a circular double stranded DNA genome (Bernard et al., 2010) ranging from 6953 bp (*Chelonia mydas* papillomavirus type 1) to 8607 bp (Canine papillomavirus type 1 (CPV1)) in length (Van Doorslaer, 2013). Typically, the genome contains six canonical genes that are located in 2 regions: genes coding for early proteins (E1, E2, E6 and E7, functional proteins) and genes encoding late proteins (L1 and L2, capsid proteins). The non-coding region is required for control of replication and transcription. All these genes are expressed in a temporal and highly regulated manner (Bernard et al., 2010; Howley and R., 2006). Generally, papillomaviruses (PVs) infect the stratified squamous epithelium of the skin and of the mucosa in various vertebrate species persisting asymptomatically or causing neoplasia (Van Ranst et al., 1992b). The host range of PVs expands across a wide array of vertebrates and PVs have been characterized from humans, domestic and wild mammals and also in birds and reptiles (turtle and snake) (Rector and Van Ranst, 2013). Recently

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bats have come to the limelight as an important reservoir of mammalian viruses including very pathogenic ones like Severe Acute Respiratory Syndrome (SARS), Middle East Respiratory Syndrome (MERS)-related coronaviruses, as well as *Filoviridae*, such as Marburgvirus, and Henipaviruses. Despite the identification and characterization of several novel viruses infecting bats, only a few PVs have been completely sequenced. Our group sequenced the first bat PV (RaPV1) from an Egyptian fruit bat *Rousettus aegyptiacus* (Rector et al., 2006) and thereafter PVs were isolated from a Rickett's bigfooted bat *Myotis ricketti* (MrPV1) and a Schreiber's long-fingered bat *Miniopterus schreibersii,* (MscPV1, MscPV2) (Tse et al., 2012; Wu et al., 2012). The most recent ones (EhPV1, from *Eidolon helvum [Eh]*; EsPV1, EsPV2, and EsPV3, from *Eptesicus serotinus*; and RfPV1, from *Rhinolophus ferrumequinum*) were sequenced and analyzed by García-Pérez (Garcia-Perez et al., 2013, 2014), whereas Wang and colleagues (2015) identified two novel divergent incomplete PV sequences from New Zealand in lesser short-tailed bats *Mystacina tuberculate*. Also, Baker and colleagues identified two partial sequences of papillomaviruses isolated from *Eidolon helvum* from Ghana (Baker et al., 2013). Most of these bat PVs are distantly related to each other and therefore belong to different phylogenetic clades (genera). De Villiers and colleagues (2004) established the criteria for the classification of PVs: different genera share less than 60% nucleotide sequence identity and species within a genus share between 60% and 70% nucleotide (nt) identity of the ORF L1 (Garcia-Perez et al., 2014; Mengual-Chulia et al., 2012).

Here, we describe the complete sequences of two novel divergent PVs (*Eidolon helvum papillomavirus 2* [EhPV2] and *Eidolon helvum papillomavirus 3* [EhPV3]) and three partially sequenced PVs (BATPV61, BATPV890a and BATPV890b) obtained from the fecal samples of straw-colored fruit bats *Eidolon helvum* in Cameroon. The partial genome analyses also showed that they could be new species of PVs.

2. Methods

2.1. Sample collection

Bat fecal samples were collected between December 2013 and May 2014 by a previously described method developed by Donaldson and colleagues (2010), after obtaining an authorization from the Delegation of Public Health for South West Region. Briefly, bats were captured in 3 different regions (Lysoka, Muyuka and Limbe) of the South West Region of Cameroon using mist nets around fruit trees and around human dwellings. Captured bats were retrieved from the net traps and held in paper sacks for 10 to 15 min, allowing enough time for the excretion of fresh fecal boluses. Sterile disposable spatulas were used to retrieve feces from the paper sacks, and placed into tubes containing 1 ml of universal transport medium (UTM). Labeled samples were put on ice and then transferred to the Cell and Molecular biology Laboratory, Biotechnology Unit, University of Buea, Cameroon and stored at −20 °C, until they were shipped to the Laboratory of Clinical and Epidemiological Virology, Leuven, Belgium where they were stored at −80 °C.

2.2. Sample preparation and sequencing

To reduce the sequencing cost, three to five fecal samples were pooled (87 samples and 25 pools in total), and treated to enrich viral particles using the previously described NetoVIR protocol (Conceicao-Neto et al., 2015): fecal suspensions (10% w/v in universal transport medium) were homogenized for 1 min at 3000 rpm with a MINILYS homogenizer (Bertin Technologies) and filtered using a 0.8 μm PES filters (Sartorius). The filtrate was then treated with a cocktail of Benzonase (Novagen) and Micrococcal Nuclease (New England Biolabs) at 37 °C for 2 h to digest free-floating nucleic acids. Samples were extracted using the QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions but without addition of carrier RNA to the lysis buffer. First and second strand synthesis and random PCR amplification for 17 cycles were performed using a slightly modified Whole Transcriptome Amplification (WTA2) Kit procedure (Sigma-Aldrich), with a denaturation temperature of 95 °C instead of 72 °C to allow for the denaturation of dsDNA and dsRNA. WTA2 products were purified with MSB Spin PCRapace spin columns (Stratec) and the libraries were prepared for Illumina sequencing using a modified version of the NexteraXT Library Preparation Kit (Illumina). Sequencing of the samples was performed on a HiSeq 2500 platform (Illumina) for 300 cycles (2 × 150 bp paired ends).

PV reads were identified in 10 pools out of 25. PCR primers (Supplementary data S1) were designed to identify the exact sample(s) in which the PVs were present. After identification of the samples of interest, the PV genomes were then amplified using the multiply-primed rolling circle amplification (RCA) method with the TempliPhi 100 Amplification kit (Amersham Biosciences) as previously described by Rector and colleagues (2004a, 2004b). Briefly, 1 μl of extracted DNA or water (negative control) was transferred into a 0.5 ml tube with 5 μl of TempliPhi sample buffer, containing exonuclease-protected random hexamers. The samples were denatured at 95 °C for 3 min, and afterwards placed on ice. A premix was prepared on ice by mixing for each sample 5 μl of TempliPhi reaction buffer, 0.2 μl of TempliPhi enzyme mix containing the ϕ29 DNA polymerase and exonuclease-protected random hexamers in 50% glycerol, and 0.2 μl of extra dNTPs (at a stock concentration of 25 mM of each dNTP). After mixing by vortexing, 5 μl of this premix was added to the cooled samples. The reactions were incubated overnight (approximately 16 h) at 30 °C. Afterwards, the reactions were placed on ice, subsequently heated to 65 °C for 10 min to inactivate the ϕ29 DNA polymerase, and stored at −20 °C awaiting further analysis. To investigate whether PV DNA was amplified, 2 μl of the RCA products were digested with 10 U of *Bam*HI, *Eco*RI, *Hin*dIII, *Sal*I, and *Xba*I. 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 PV DNA (~8 kb), or multiple bands with sizes adding up to this length. Furthermore, primers (Supplementary data S1) were designed and used to amplify the complete genomes with the Expand Long