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Equus asinus Papillomavirus (EaPV1) provides new insights into equine papillomavirus diversity

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ABSTRACT

We detected a novel papillomavirus (EaPV1) from healthy skin and from sun associated cutaneous lesions of an Asinara (Sardinia, Italy) white donkey reared in captivity in a wildlife recovery centre. The entire genome of EaPV1 was cloned, sequenced, and characterised. Genome is 7467 bp long, and shows some characteristic elements of horse papillomaviruses, including a small untranslated region between the early and late regions and the lack of the retinoblastoma tumour suppressor binding domain LXCXE in E7. Additionally, a typical E6 ORF is missing. EaPV1 DNA was detected in low copies in normal skin of white and grey donkeys of the Asinara Island, and does not transform rodent fibroblasts in standard transformation assays. Pairwise nucleotide alignments and phylogenetic analyses based on concatenated E1-E2-L1 amino acid sequences revealed the highest similarity with the Equine papillomavirus type 1. The discovery of EaPV1, the prototype of a novel genus and the first papillomavirus isolated in donkeys, confirms a broad diversity in *Equidae* papillomaviruses. Taken together, data suggest that EaPV1 is a non-malignant papillomavirus adapted to healthy skin of donkeys.

1. Introduction

The *Papillomaviridae* family consists of a large and diverse group of viruses characterised by a double strand, covalently linked circular genome typically ranging from 7 to 8 kb in size, and causing proliferative lesions in animals and human (Howley and Lowy, 2007). Based on DNA sequence homologies, about 200 different human papillomavirus types (HPVs) have been identified and classified into a number of genera (Bernard et al., 2010; Rector and Van Ranst, 2013). According to their association with cancer, HPVs of the *alpha* genus (α-HPVs) are classified as low-risk, such as types 6 and 11, and as high-risk, such as types 16 and 18 (Muñoz et al., 2003; Howie et al., 2011). While low-risk α-HPVs have been associated to benign proliferative lesions, high-risk α-HPVs can cause cervical, anogenital, and head and neck cancer (zur Hausen, 1999; Cogliano et al., 2005). Lately, HPVs of the beta genus (β-HPVs) are being increasingly investigated due to a possible role in development of nonmelanoma skin cancer (Howley and Lowy, 2007; Haedicke and Iftner, 2013). As a general principle, it is

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accepted that PVs sharing less than 60% nucleotide identity in the L1 ORF represent different genera. Similarly, nucleotide identities below 70% and 90% define different species and types, respectively (De Villiers et al., 2004; Bernard et al., 2010). Non-human PVs have been detected in normal skin and in both benign and malignant proliferative lesions of 54 different animal species (Terai et al., 2002; Alberti et al., 2010; Rector and Van Ranst, 2013; Scagliarini et al., 2013). In single animal species a genotype diversity comparable to that found in humans has not yet been identified, even if there was an exponential increase in the number of animal papillomaviruses identified over the last decade (Rector and Van Ranst, 2013). The association of animal papillomavirus types to defined degrees of pathogenicity needs to be highlighted, the suspected great diversity of animal PVs still remaining uncovered. Indeed, each single animal species potentially hosts a number of PV types comparable to the one found in human.

Based on the number of types identified in single animal species, the most widely studied animal PV infections are those of domestic animals (Lange et al., 2013b). According to the PapillomaVirus Episteme (PaVE, http://pave.niaid.nih.gov/#home), 13 viruses have been found and fully sequenced in dogs, 12 in cattle (Zhu et al., 2012), 7 in horses (Lange et al., 2013b), 3 in sheep (Alberti et al., 2010) and cats (Munday et al., 2013).

Horse PVs are often quoted as an example of exception to Clay's rule, according to which related PVs infect phylogenetically related host species (Gottschling et al., 2011). Indeed, horses are commonly infected by bovine PVs type 1 and 2 (BPV1, BPV2), which are the causative agents of equine sarcoid (Nasir and Campo, 2008). Apart from BPV1 and BPV2, the seven horse papillomaviruses (EcPV1 to 7) therefore represent the PV diversity so far described in *Equidae*.

Equidae PVs seem to be associated to distinct clinical conditions (Lange et al., 2013a,b). EcPV1 has been isolated from benign proliferative lesions of young horses (Postey et al., 2007). EcPV2, EcPV3, EcPV4, EcPV5, EcPV6, and EcPV7 were found both in benign and malignant genital neoplasia, in aural plaques, in semen and in apparently healthy skin, some of them often in the same host (Lange et al., 2011, 2013a,b; Kainzbauer et al., 2012; Sykora et al., 2012). The absence of closely related viruses complicates the taxonomic and phylogenetic allocation of *Equidae* PVs. In a recent work conducted to quantify the phylodynamic forces driving PVs evolution, Gottschling et al. (2011) obtained significant associations of Delta + Zeta-PVs with *Perissodactyla* and *Ruminantia*, with the two horse PVs EcPV1 and EcPV2 at the root of these lineages.

Aim of this study was to investigate papillomavirus diversity in *Equidae* and to establish the presence of PV in species related to horse. We identified EaPV1, the first papillomavirus found in donkey. By means of molecular and bioinformatics tools we fully characterised EaPV1 genome, described its main features at the nucleotide and amino acid levels, and investigated its phylogeny and evolution. The ability of the EaPV1 early region to transform rat fibroblasts in standard transformation assay and the viral loads in clinically healthy skin of a number of animals living in close contact were also investigated.

2. Material and methods

2.1. Animals and samples

During a survey conducted to identify cutaneous PVs in wild and domestic animals, swabs were collected from normal skin and from skin presenting changes compatible with solar damage (erythema, scales, and dark pigmented macules) of an Asinara white donkey identified as EA01M (Fig. 1A), and hosted in the Wildlife Recovery Centre of Bonassai (Sardinia, Italy). The Asinara donkey (*Equus asinus* var. albina) is a rare breed of feral white donkey indigenous to the island of Asinara, which lies off the Northwest coast of Sardinia, Italy. It is one of the 'seven indigenous donkey breeds of limited distribution' listed by the Italian breeders association. A population of around 120 animals is actually free-ranging on the Asinara island National Park, while a smaller group of around 30 animals lives in the Porto Conte Regional Park (Northern Sardinia). All these animals are characterised by a white coat, pink or light blue eyes, due to incomplete albinism, and a small size (around 1 m height).

Full-thickness skin specimens were obtained from both the right and left pinna of donkey EA01M by punch biopsy (Fig. 1B). Tissues were fixed in 10% neutral-buffered formalin, embedded in paraffin, and sectioned to 4–6 μm slices. Adjacent slices were mounted onto glass slides and stained with haematoxylin and eosin (H&E) for light microscopy. A second set of tissues was kept at −80 °C until nucleic acids extraction. Cutaneous swabs were also sampled from 10 white and 8 grey free-living donkeys in the Asinara Island. All donkeys were subjected to dermatological and physical examination.

2.2. Nucleic acid extraction and PV genome investigation

DNA was extracted from punch biopsies and skin swabs respectively using the DNeasy Blood & Tissue Kit and the QIAamp DNA Mini Kit (Qiagen, Italy), following manufacturer recommendations. To identify PV genomes, the rolling circle amplification (RCA) technique combined with restriction enzyme digestion was performed. RCA was carried out by using the TempliPhi 100 Amplification Kit (GE Healthcare, Italy), following the protocols previously described by Rector et al. (2004) and modified in Alberti et al. (2010). Briefly, 5 μl of DNA extractions were mixed with 10 μl of sample buffer and subsequently heated for 3 min at 95 °C, then transferred on ice. Ten microliters of TempliPhi reaction buffer, 0.4 μl of TempliPhi enzyme mix containing phi 29 DNA polymerase, random hexamers in 50% glycerol, and 0.4 μl of 10 mM dNTPs per sample were mixed and added to the cooled sample. The final reaction was subsequently incubated for 16 h at 30 °C. Phi 29 polymerase was eventually inactivated at 65 °C for 10 min. RCA products were digested with restriction enzymes EcoRI, BamHI and HindIII and run on a 0.8% agarose gel to visualize the presence of a DNA profile consistent with the length of a papillomaviral genome, or of multiple bands with