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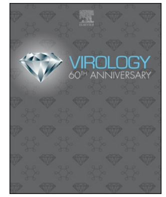

Generation of a novel next-generation sequencing-based method for the isolation of new human papillomavirus types

Rosario N. Brancaccio[a,1], Alexis Robitaille[a,1], Sankhadeep Dutta[a], Cyrille Cuenin[a], Daiga Santare[b], Girts Skenders[b], Marcis Leja[b], Nicole Fischer[c,d], Anna R. Giuliano[e], Dana E. Rollison[e,f], Adam Grundhoff[d,g], Massimo Tommasino[a,*], Tarik Gheit[a,*]

[a] International Agency for Research on Cancer, Lyon, France
[b] Institute of Clinical and Preventive Medicine, University of Latvia, Riga, Latvia
[c] Institute for Medical Microbiology, Virology and Hygiene, University Medical Center Hamburg-Eppendorf, Hamburg, Germany
[d] German Center for Infection Research, partner site Hamburg, Borstel, Lübeck, Riems, Germany
[e] Center for Infection Research in Cancer, Moffitt Cancer Center, Tampa, FL, USA
[f] Department of Cancer Epidemiology, Moffitt Cancer Center, Tampa, FL, USA
[g] Heinrich-Pette Institute, Leibniz Institute for Experimental Virology, Hamburg, Germany

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ABSTRACT

With the advent of new molecular tools, the discovery of new papillomaviruses (PVs) has accelerated during the past decade, enabling the expansion of knowledge about the viral populations that inhabit the human body. Human PVs (HPVs) are etiologically linked to benign or malignant lesions of the skin and mucosa. The detection of HPV types can vary widely, depending mainly on the methodology and the quality of the biological sample. Next-generation sequencing is one of the most powerful tools, enabling the discovery of novel viruses in a wide range of biological material. Here, we report a novel protocol for the detection of known and unknown HPV types in human skin and oral gargle samples using improved PCR protocols combined with next-generation sequencing. We identified 105 putative new PV types in addition to 296 known types, thus providing important information about the viral distribution in the oral cavity and skin.

1. Introduction

Human papillomaviruses (HPVs) are non-enveloped viruses with double-stranded circular DNA of about 8 kb that can colonize the mucosal and cutaneous epithelia (Bernard et al., 2010; Bzhalava et al., 2013). To date, more than 200 PVs have been isolated from different body sites and fully characterized, and this number continues to grow (Bzhalava et al., 2015; Smelov et al., 2017). Based on the nucleotide sequences of the major capsid protein L1, HPVs are classified into genera, species, and types (Bernard et al., 2010). HPV types are organized into five major genera: alpha, beta, gamma, mu, and nu (de Villiers et al., 2004). The genera alpha, beta, and gamma include the majority of the known HPVs. The alpha HPV types have been extensively studied, because of their clear association with human carcinogenesis (Tommasino, 2014). The high-risk (HR) HPV group includes at least 12 HPV types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59), which are the etiological agents of anogenital cancers and a subset of head and neck cancers, particularly oropharyngeal cancer (Bouvard et al., 2009; Haedicke and Iftner, 2013). The genus alpha also includes the low-risk HPV types (HPV6 and 11) that are associated with benign genital lesions and with laryngeal disease in children (Giuliano et al., 2008b; Goon et al., 2008).

The genus beta includes approximately 50 different HPV types, fully characterized, that are subdivided into five species (beta HPV species 1–5). The majority of the beta HPV types belong to species beta-1 and beta-2 and are widely present in the skin of healthy individuals. Only 7 HPV types have been classified into the species beta-3 ($n = 4$), beta-4 ($n = 1$), and beta-5 ($n = 2$). HPV types of genus beta can induce warts and have been associated with certain forms of non-melanoma skin carcinoma (NMSC) (Orth, 2006). The first beta HPVs, HPV5 and 8, were isolated from the skin of patients with epidermodysplasia verruciformis (EV), a rare autosomal recessive hereditary skin disorder that confers high susceptibility to beta HPV infection and cutaneous squamous cell carcinoma development at sun-exposed regions (Pfister, 2003). Several studies showed that beta HPV types are associated with NMSC development in non-EV individuals (Andersson et al., 2008; Berkhout et al.,

* Corresponding authors.
E-mail addresses: icb@iarc.fr (M. Tommasino), gheitt@iarc.fr (T. Gheit).
[1] These authors contributed equally to this work.

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2000; Bouwes Bavinck et al., 2010; Casabonne et al., 2007; Cornet et al., 2012; de Jong-Tieben et al., 1995; Harwood et al., 2000; Iannacone et al., 2014; Iftner et al., 2003; Karagas et al., 2006; Waterboer et al., 2008). Patients with a history of NMSC show elevated positivity for markers of beta HPV infection compared with healthy individuals (Ally et al., 2013; Asgari et al., 2008; Iannacone et al., 2012). Recent studies reported the presence of beta HPV types at additional anatomical sites other than the skin, such as the oral mucosal epithelium, eyebrow hairs, penile and external genital samples, and the anal canal (Arroyo et al., 2013; Barzon et al., 2011; Donà et al., 2016; Pierce Campbell et al., 2016; Smelov et al., 2017).

Species beta-3 HPV types appear to have a dual tropism, being present in the skin and the mucosal epithelia (Forslund et al., 2013; Hampras et al., 2017). Interestingly, studies in *in vitro* and *in vivo* experimental models have highlighted some biological similarities between beta-3 HPV and mucosal HR HPV types (Cornet et al., 2012; Viarisio et al., 2016). In addition, Viarisio et al. (2016) showed that beta-3-HPV49 transgenic mice were highly susceptible to upper digestive tract carcinogenesis upon initiation with 4-nitroquinoline 1-oxide.

HPVs from the gamma, mu, and nu genera induce cutaneous papillomas or warts (de Villiers et al., 2004) and have been poorly investigated so far. To date, approximately 80 different gamma HPV types have been isolated from the skin and genital tract (retrieved from GenBank, September 2017).

In addition to the fully characterized HPV types, a substantial number of partial genomic sequences of putative novel HPV types have been deposited to GenBank, indicating that many more HPV types exist. So far, the molecular biology techniques for the isolation of novel HPV types have been based mainly on the use of degenerate and/or consensus primers, followed by cloning and Sanger sequencing (Chouhy et al., 2010; Forslund et al., 1999). However, considering the large number of recently characterized HPV genomes, degenerate primers may be improved in order to discover novel HPV types. In particular, this strategy may lead to the expansion of species that so far include a very small number of HPV types, such as species beta-3 ($n = 4$), beta-4 ($n = 1$), and beta-5 ($n = 2$).

In this study, we used novel and well-validated consensus and degenerate primers to amplify genomic HPV sequences from human DNA isolated from oral and skin specimens. Analysis of the PCR products by next-generation sequencing (NGS) resulted in the identification of 105 putative new PV types.

2. Materials and methods

2.1. Sample collection and DNA extraction

Skin swabs and oral rinses from two different ongoing studies aiming to determine the prevalence of viral DNA and its associations with disease were used in the present analysis (Hampras et al., 2014, 2015; Nunes et al., 2016; Pierce Campbell et al., 2013, 2016).

Skin swab specimens ($n = 119$) were randomly selected from the VIRUSCAN Study, an ongoing five-year (2014–2019) prospective cohort study conducted at Moffitt Cancer Center and the University of South Florida (R01CA177586-01; “Prospective study of cutaneous viral infections and non-melanoma skin cancer”). An area of approximately 5 × 5 cm of the top of the sun-exposed forearm was sprayed with 0.9% saline solution. A cotton-tipped Dacron swab (Digene, Gaithersburg, MD, USA) was then rubbed back and forth a few times to collect exfoliated skin cells. Individual swabs were placed in a separate vial and preserved in Digene Standard Transport Medium.

In addition, 62 oral rinses were randomly selected from the HPV Infection in Men (HIM) study, a large, multinational (Brazil, Mexico, and the USA) prospective cohort study of the natural history of HPV infection in men. The HIM study methods have previously been described in detail (Giuliano et al., 2008a, 2009, 2011; Nyitray et al., 2011). A further 85 oral samples were selected from a pilot study that aimed to estimate the prevalence of *Helicobacter pylori* in oral gargles from a Latvian population. The study was approved (No. 8-A/15) by the Ethics Committee of Riga East University Hospital Support Foundation.

After DNA extraction, all samples were analyzed at the International Agency for Research on Cancer (Lyon, France) for viral DNA from HPV.

Table 1
Sequences of the oligonucleotides and composition of the different protocols. i = inosine; W = A or T; D = A or G or T; K = T or G; Y = C or T; M = A or C; R = A or G; V = A or C or G; H = A or C or T.

Primer mix	Primer sequence (5–3′)
Beta-3-1	
B3L1FW3	AGGACATCCATACTTTGAGGTTCGAG
B3L1FW4	TAGGACATCCATATTTTGATGTGAGAG
B3L1FW5	GATGTTAGAGACACTGGAGATTCAACA
B3L1FW6	GATGTTAGAGACACTGGGGATTCAACA
B3L1FW7	GATGTTAGAGACACTGTGGATCAAACA
B3L1RW	ATAATAGTATTTCTTAATTCTAATGGAGG
B3L1RW4	ATAACTGAATTGATTAATTCTAATGGAGG
B3L1RW5	ATAACTGTATTTACTAATTCTAAAGGTGG
B3L1RW6	TACAGTATTTACCAGTTCCAAAGGTGG
B3L1RW7	ATTACAGTATTAACTAATTCTAAAGGTGG
B3L1RW8	ATTACAGTATTTACTAATTCTAAAGGTGG
Beta-3-2	
B3L1FW1	GTAGGACATCCATAYTTTGAKGTKiGAG
B3L1FW2	TTGATGTTAGAGACACTGiDGATYMAACA
B3L1RW1	ATAAiWGWATTKYTTAATTCTAATGGAGG
B3L1RW2	ATTACAGTATTiACKARTTCYAAAGGTGG
CUT	
CUT1Fw	TRCCiGAYCCiAATAARTTTG
CUT1AFw	TRCCiGAYCCiAACAGRTTTG
CUT1BFw	TRCCiGAYCCiAATAGRTTTG
CUT1CFw	TRCCiGAYCCiAACAARTTTG
CUT1BRv	ARGAYGGiGAYATGGTiGA
FAP	
FAP59	TAACWGTiGGiCAYCCWTATT
FAP64	CCWATATCWVHCATiTCiCCATC
FAPM1	
FAP59.1	TAACAGTDGGiCAYCCWTWTT
FAP59.2	TAACAGTDGGiCAYCCWTAYT
FAP64.1	CCDATATCWVHCATATCiCCATC
FAP59	TAACWGTiGGiCAYCCWTATT
FAP64	CCWATATCWVHCATiTCiCCATC
FAPM2	
FAP59.2	TAACAGTDGGiCAYCCWTAYT
FAP64.1	CCDATATCWVHCATATCiCCATC

2.2. PCR protocols

The following PCR protocols using different sets of primers were run (Table 1): (i) CUT primers, as previously described (Chouhy et al., 2010); (ii) FA-type (FAP) primers, as previously described (Forslund et al., 1999); (iii) a new set of FAP primers, i.e. FAP59.1, FAP59.2, and FAP64.1 (Fig. 1; Table 1); these primers were used to generate two different primer mixtures (FAPM1 and FAPM2); the PCR conditions were the same as for the original FAP protocol; (iv) a set of 11 beta-3 specific primers (henceforth referred to as beta-3-1) (Table 1); and (v) a set of 4 broad-spectrum beta-3 degenerate primers (henceforth referred to as beta-3-2). The beta-3-1 and beta-3-2 primers were synthesized by MWG Biotech (Ebersberg, Germany) and mixed to obtain a 10 × solution containing 2 μM of each primer. PCR was performed with the Qiagen Multiplex PCR kit (Hilden, Germany) according to the manufacturer's instructions. The use of these primers enables the amplification of a region in the L1 gene of approximately 450 bp.

2.3. Validation of the new set of primers

To evaluate the sensitivity of the novel HPV PCR protocols (beta-3-1, beta-3-2, FAPM1, and FAPM2), we used an artificial mixture containing cloned HPV genomes at different relative concentrations (10-

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