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# High prevalence of *Gammapapillomaviruses* (*Gamma*-PVs) in pre-malignant cutaneous lesions of immunocompetent individuals using a new broad-spectrum primer system, and identification of HPV210, a novel *Gamma*-PV type

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ABSTRACT

Genus *Gammapapillomavirus* (*Gamma*-PV) is the most diverse and largest clade within the *Papillomaviridae* family. A novel set of degenerate primers targeting the E1 gene was designed and further used in combination with the well-known CUT PCR assay to assess HPV prevalence and genus distribution in a variety of cutaneous samples from 448 immunocompetent individuals. General HPV, *Gamma*-PV and mixed infections prevalence were significantly higher in actinic keratosis with respect to benign and malignant neoplasms, respectively ($p = 0.0047$, $p = 0.0172$, $p = 0.00001$). *Gamma*-PVs were significantly more common in actinic keratosis biopsies than *Beta*- and *Alpha*-PVs ($p = 0.002$). The full-length genome sequence of a novel putative *Gamma*-PV type was amplified by 'hanging droplet' long-range PCR and cloned. The novel virus, designated HPV210, clustered within species *Gamma*-12. This study provides an additional tool enabling detection of HPV infections in skin and adds new insights about possible early roles of *Gamma*-PVs in the development of cutaneous malignant lesions.

## 1. Introduction

Papillomaviruses (PVs) are a highly diverse group of non-enveloped and double stranded DNA viruses that possibly infect mucosal and cutaneous epithelia of all vertebrates (de Villiers et al., 2004; Bravo et al., 2010). Classification of PVs is based on phylogenetic relationships of their complete L1 gene sequences (de Villiers et al., 2004). Currently, 324 human papillomavirus (HPV) types have been described: 221 officially recognized from the International Human Papillomavirus Reference Center at the Karolinska Institutet (Stockholm, Sweden; http://www.nordicehealth.se/hpvcenter/reference_clones) and 103 identified only by Next Generation Sequencing (NGS) techniques and available at the Papillomavirus Episteme (https://pave.niaid.nih.gov; Van Doorslaer et al., 2017). HPVs are grouped within five (*Alpha*, *Beta*, *Gamma*, *Mu* and *Nu*) out of more than 30 PV genera recognized to date, and further classified into mucosal/genital and cutaneous HPV types, based on the clinical manifestation of infections.

Cutaneous HPVs are distributed over all five HPV genera and represent approximately 75% of all HPV types described to date (de Villiers, 2013). In contrast to mucosal high-risk HPV types, clustering to the *Alpha*-PV genus, which have been recognized as oncogenic by WHO (WHO/ICO, 2010), the association of specific cutaneous HPV types with skin carcinogenesis remains to be demonstrated (Quint et al., 2015). Nevertheless, the relationship between *Beta*-PV type infections and

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devolvement of non-melanoma skin cancer (NMSC; basal and squamous cell carcinomas) has been established in patients with epidermodysplasia verruciformis (Nindl et al., 2007; Lazarczyk et al., 2009). Moreover, *Beta*-PV DNA has been detected in a high proportion of cutaneous squamous cell carcinomas and their precursor lesions, actinic keratosis, in both organ-transplant recipients (Feltkamp et al., 2008; Proby et al., 2011) and immunocompetent individuals (Chahoud et al., 2016). Epidemiological data have shown that one in 100–1000 skin tumor cells harbor *Beta*-PV genomes, with higher prevalence and viral loads in actinic keratosis than in squamous cell carcinomas (Pfister et al., 2003; Weissenborn et al., 2005). These observations, together with molecular and histopathological data (Quint et al., 2015), support the current model of *Beta*-PV types as co-factors, together with UV-radiation, in the early pathogenesis of skin cancer (Pfister, 2003; Akgül et al., 2006; Nindl et al., 2007). However, the etiological role of cutaneous HPV infection and the mechanism(s) involved in skin carcinogenesis remain to be further elucidated (Howley and Pfister, 2015; McLaughlin-Drubin, 2015; Quint et al., 2015).

*Gamma*-PV genus is the most diverse and largest clade within the family *Papillomaviridae*, with 185 completely sequenced HPV types, surpassing *Alpha*- and *Beta*-PV genera, with 66 and 65 HPV types, respectively (Van Doorslaer et al., 2017). Improved molecular methods used for detection of cutaneous HPVs have led to the identification of many novel *Gamma*-PV types in common warts and other skin tumors (Bzhalava et al., 2013; Ekström et al., 2013; Hošnjak et al., 2015). Recent data have shown that HPV197 (species *Gamma*-24) is the most commonly detected HPV type in squamous cell carcinomas (Arroyo Muhr et al., 2015). Moreover, it has been demonstrated that six out of nine HPV types/putative types (HPV158, FA9, GC05, KC45, SE126 and SE253), belonging to the *Gamma*-PV genus, are by far more abundant (> 10-fold) in pooled samples of squamous cell carcinomas and actinic keratosis, in comparison to keratoachantomas (Bzhalava et al., 2014). Nevertheless, so far there is no evidence that *Gamma*-PVs contribute to the development of NMSC in immunosuppressed or immunocompetent individuals.

Since *Gamma*-PV genus is much more diverse than previously known (Van Doorslaer et al., 2017) and among 193 distinct commercial HPV tests currently available for detection of HPV infection, all but two target *Alpha*-PV types only (Poljak et al., 2016), novel detection methods are needed to establish tissue tropism and potential clinical relevance of novel and previously known *Gamma*-PVs. In the present study we describe the design of a novel primer system which enables the amplification of HPV types clustering to the *Gamma*-PV genus (*Gamma*-PV PCR). *Gamma*-PV PCR was further used in a combination with the well-known CUT PCR assay (Chouhy et al., 2010; Brancaccio and Robitaille et al., 2018) in order to assess the HPV prevalence and genus distribution in a variety of cutaneous samples from immunocompetent individuals. Additionally, we report the identification of 15 novel putative HPV types and the complete molecular and phylogenetic characterization of HPV210, a novel HPV type clustering to the species *Gamma*-12.

## 2. Materials and methods

### 2.1. Patients' data, sample collection and processing

Overall, 653 samples from cutaneous epithelia, obtained from 448 immunocompetent individuals [227 males and 221 females, median age: 55 years (range, 16–92 years)] were analyzed in this study (Table 1).

All subjects provided written informed consents for participation in the study and were interviewed about their demographic characteristics (age, gender, place of birth), history of skin pathologies and family history of skin cancer. All enrolled patients were examined by a dermatologist and all lesions were additionally subjected to histopathological evaluation at the División de Anatomía Patológica, Facultad de Ciencias Médicas, Universidad Nacional de Rosario, Argentina. The study was conducted according to the Declaration of Helsinki and was approved by the Institutional Review Board of the Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Rosario, Argentina (reference number 6060/134).

Eyebrow hair follicles, healthy forehead skin swabs, lesion swabs and lesion biopsies were collected, processed, and stored at − 80 °C until further analysis, as described previously (Kocjan et al., 2005; Chouhy et al., 2010; Bolatti et al., 2017). The adequacy of swab and biopsy samples for downstream analyses was determined by PCR amplification of the human beta-globin gene (Saiki et al., 1986).

### 2.2. Gamma-PV PCR primer design

Nucleotide sequences of the E1 open reading frame (ORF) of 64 completely characterized *Gamma*-PV types, available at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/), were aligned using the ClustalW algorithm, provided within the MEGA6 software package (Tamura et al., 2013). Based on the alignment, several primer candidates, with a conserved sequence of 8–10 nucleotides at the 3′ region and a degenerate 5′ region, were found and subsequently analyzed for primer-dimer and primer-hairpin formations. Finally, two primers showing the lowest number of mismatches at the 3′ region were selected: FFPE1-Fw (5′-twiywghiytaaaacgaaagt-3′; i = inosine) and FFPE3-Rv (5′-sawwagwatytkcagyttcat-3′). The alignment of the 64 *Gamma*-PV types used to design the *Gamma*-PV primer system and the positions of the primer binding regions is shown in Supp. Table 1. The positions of the forward and reverse primer corresponded to nucleotides 1033-1053 and 1191-1171, respectively, of the HPV4 complete genome sequence (GenBank accession No. X70827), yielding an amplicon of 158 bp.

### 2.3. Determination of HPV infection in clinical samples

The presence of HPV infection was determined using the newly designed *Gamma*-PV PCR and an improved version of the original CUT PCR assay targeting L1 ORF, using modified primers. Briefly, CUT primers were originally designed using an L1 ORF nucleotide sequence alignment derived from 88 completely characterized cutaneotropic and mucosotropic HPV types from *Alpha*-, *Beta*-, *Gamma*-, *Mu*- and *Nu*-PV genera (Chouhy et al., 2010). Subsequently, the CUT PCR primer system was improved with the design of 3 novel primers [CUT1EFw (5′-trccigaycciaatagatttg-3′; i = inosine), CUT1CRv (5′-tcicacatrtciccrtcytg-3′; i = inosine) and CUT1DRv (5′-tcisccatrtciccrtcytg-3′; i = inosine)] based on the alignment of L1 ORF sequences of 244 additional HPV types/putative types. Furthermore, the original primers CUT1AFw, CUT1BFw and CUT1CFw (Chouhy et al., 2010) were excluded from the improved CUT PCR, based on the specificity analysis, as in some conditions these primers enabled the amplification of human genomic DNA. Current CUT primer-binding regions within the alignment of L1 ORFs of 64 Gamma-PVs are shown in Suppl. Table 1.

All PCR reactions were performed using the thermocycler Mastercycler Personal (Eppendorf, Hamburg, Germany).

*Gamma*-PV PCR was performed in a reaction mixture with a final volume of 25 µl, containing 3 µl of sample DNA. Reaction mixtures contained 0.8 µM of each primer, 150 µM of each dNTP (Thermo Scientific, Waltham, USA), 4 mM of $MgCl_2$, 0.2% BSA, 1 X PCR buffer with $(NH_4)_2SO_4$ and 2.5 U of Taq DNA polymerase (Thermo Scientific). Reaction mixtures were first heated for 2 min at 94 °C, followed by 5 cycles of 20 s at 94 °C, 20 s at 50 °C and 10 s at 66 °C, 10 cycles of 20 s at 94 °C, 20 s at 49 °C and 10 s at 66 °C, 10 cycles of 20 s at 94 °C, 20 s at 48 °C and 10 s at 66 °C, 20 cycles of 20 s at 94 °C, 20 s at 47 °C and 10 s at 66 °C, followed by 2 min at 66 °C.

CUT PCR was performed in a reaction mixture with a final volume of 25 µl, containing 5 µl of sample DNA. Reaction mixtures contained 0.4 µM of primer CUT1Fw, 0.4 µM of primer CUT1EFw, 0.266 µM of

**Table 1**
Characteristics of samples analyzed in this study.

| Histopathological diagnosis | No. of patients/volunteers | Type of samples | | | | Total no. of samples |
|---|---|---|---|---|---|---|
| | | Forehead swab | Lesion swab | Lesion biopsy | Eyebrow hair follicles | |
| Healthy skin | 188 | 111 | 0 | 0 | 77 | 188 |
| Benign skin lesions[a] | 13 | 12 | 13 | 13 | 0 | 38 |
| Seborrhoeic keratosis | 17 | 14 | 17 | 17 | 0 | 48 |
| Skin warts | 14 | 14 | 14 | 14 | 0 | 42 |
| Actinic keratosis | 71 | 0 | 71 | 70 | 0 | 141 |
| Basal cell carcinoma | 91 | 0 | 43 | 91 | 0 | 134 |
| Squamous cell carcinoma | 54 | 0 | 8 | 54 | 0 | 62 |
| **Total** | **448** | **151** | **166** | **259** | **77** | **653** |

[a] 5 soft fibromas, 1 trichilemmal cyst, 1 lichen planus, 3 dermal melanocytic nevuses, 1 hemangioma, 1 psoriasis vulgaris, 1 acantholytic acanthoma.

primer CUT1BRv, 0.266 μM of primer CUT1CRv and 0.266 μM of primer CUT1DRv, 400 μM of each dNTP (Thermo Scientific), 3.5 mM of $MgCl_2$, 1 X PCR buffer with $(NH_4)_2SO_4$ and 2.5 U of Taq DNA polymerase (Thermo Scientific). Reaction mixtures were heated for 2 min at 94 °C, followed by 40 cycles of 30 s at 94 °C, 2 s at 60 °C, followed by a ramp of 0.2 °C/s to 50 °C, 10 s at 50 °C and 40 s at 72 °C.

Following an electrophoresis in a 2.5% agarose gel, amplicons obtained with the *Gamma*-PV PCR (≈158 bp) and CUT PCR (≈370 bp) were identified by size under an UV light, and their nucleotide sequences were obtained using Sanger sequencing.

The analytical sensitivity of the *Gamma*-PV PCR was determined using a 10-fold serial dilution, spanning from 1 to 1000 HPV4 viral DNA copies per reaction, in a background of 5 ng of human placental DNA (Sigma, St. Louis, USA). For HPV4, the limit of detection of the *Gamma*-PV PCR assay was estimated to 100 copies per reaction.

### 2.4. HPV type/putative type identification and phylogenetic analyses

Nucleotide sequences derived from *Gamma*-PV and CUT PCRs were compared to all sequences available in the GenBank database (www.ncbi.nlm.nih.gov), using the Blast algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Following CUT PCR, a novel putative HPV type was identified when the fragment sequence showed less than 90% L1 ORF nucleotide identity to all officially recognized HPV types (de Villiers et al., 2004). The same criterion was also applied to classify HPV types/putative types based on partial E1 nucleotide sequences derived from the *Gamma*-PV PCR, as similar nucleotide identities among L1 and E1 ORFs were obtained in the pairwise comparison analysis of HPV types from *Alpha*-, *Beta*-, *Gamma*- and *Mu*-PV genera, respectively, as shown in the Supplementary Table 2.

Nucleotide sequences of L1 and E1 gene regions, obtained from 286 representative HPV types available at the International Human Papillomavirus Reference Center (http://www.nordicehealth.se/hpvcenter/reference_clones) and the Papillomavirus Episteme (http://pave.niaid.nih.gov), were used to construct two separated multiple sequence and pairwise alignments at the amino acid (aa) level, using the ClustalW algorithm of the MEGA6 software package (Tamura et al., 2013). Subsequently, novel putative HPV sequences derived from L1 or E1 regions were included in the mentioned alignments of representative HPV types and realigned using MAFFT´s "Align fragment sequences to an MSA" tool (Katoh and Yamada, 2017).

The phylogenetic relationships were inferred by Bayesian analysis using Beast version 1.7.5 (Drummond et al., 2012). To do so, Markov Chain Monte Carlo (MCMC) simulations were performed during $2 \times 10^7$ generations, sampling one state every 1000 generations, with a burnin of 10%. The setting "Create tree log file with branch length in substitutions" was selected to obtain the phylogram log file. The evolutionary substitution model selected for each run was GTR + I + Γ. Statistical convergence of MCMC was assessed visually by the traceplot and by calculating the effective sample size using TRACER v1.4 (available at http://beast.bio.ed.ac.uk/Tracer). The maximum clade credibility tree across all the plausible trees generated by BEAST was then computed with the TreeAnnotator program available in the BEAST package.

### 2.5. Identification of HPV210, a novel Gamma-PV type

The partial L1 nucleotide sequence of the novel HPV type, HPV210, was deposited to the GenBank database by Antonsson et al. in 2000, as the HPV isolate FA10 (GenBank Accession no. AF327732). Subsequently, our research group identified the novel HPV type in swab sample collected from a sun-exposed healthy skin of the forehead of a 53-year-old female and completely characterized the novel virus.

The complete genome sequence of the novel HPV type was obtained by generating overlapping amplicons using the 'hanging droplet' long-range PCR technique, as described previously (Chouhy et al., 2013a). While one half of the viral genome was amplified with the E1*Gamma*-2F (5′-kgghccwccagatacwgg-3′)/FAP64-R (Forslund et al., 1999) degenerate primer pair, the other half of the viral genome was obtained using a specific primer pair V48-F (5′-ccggtgatggggcagaac-3′)/V48-R (5′-caaaacgggcggtcagtgt-3′). Specific primers were designed based on the 3′ and 5′ regions of the half of the viral genome obtained with the E1*Gamma*-2F/FAP64-R generic primers as a template, using the FastPCR program (Kalendar et al., 2014). Reaction mixtures and cycling conditions were the same as described previously (Bolatti and Chouhy et al., 2016), using 50 °C as an annealing temperature for the V48-F/V48-R primer pair in both rounds of amplification.

The amplicons generated with generic and specific primers (each approximately 4 kbp in lenght) were excised from the agarose gel, purified with NucleoSpin Extract II kit (Macherey-Nagel, Germany), and cloned with the pGEM-T Easy Cloning kit (Promega, US) and TOPO XL PCR Cloning Kit (Invitrogen, US), respectively. Each viral genomic half was fully sequenced by primer walking method on both DNA strands and the obtained consensus sequence was compared to the available nucleotide sequences in the GenBank database, using the Blast algorithm.

DNA clones and the corresponding nucleotide sequences were subsequently submitted to the International HPV Reference Center in August 2015 for official designation of the novel HPV type number (HPV210). Reconfirmation of the nucleotide sequences was obtained in February 2016. The phylogenetic analysis of HPV210 was performed by Bayesian analysis as described above, using the alignment of HPV210 L1 ORF with 286 representative HPV types.

### 2.6. Molecular characterization of HPV210

The ORFs of HPV210 were determined using the ORF finder tool of the SnapGene software v1.1.3 (GSL Biotech LLC). In addition, viral genomic regions and protein's functional domains were characterized in detail using previously published data (Kovanda et al., 2011; Chouhy et al., 2013a; Oštrbenk et al., 2015; Bolatti et al., 2016).

### 2.7. Statistical analysis

The statistical analyses of categorical variables were performed by Chi square test, Fisher's exact test, McNemar's test and Cochran's test. *p* values below 0.05 were regarded as statistically significant.

### 2.8. Nucleotide sequence accession numbers

The GenBank/EMBL/DDBJ accession numbers for the novel HPV type/putative types reported in this paper are: EP06 (MH460941), EP07 (MH460942), EP08 (MH460943), EP10 (MH460944), EP13 (MH460945), EP15 (MH460946), EP16 (MH460947), EP17 (MH460948), EP18 (MH460949), EP19 (MH460950), EP20 (MH460951), EP21 (MH460952), EP22 (MH460953), EP23 (MH460954), EP24 (MH460955), HPV210 (MH460956).

## 3. Results

### 3.1. HPV detection and typing using Gamma-PV and CUT PCRs

Overall, 112 different HPV types/putative types (14 *Alpha*-, 30 *Beta*-, 67 *Gamma*-, and 1 *Mu*-PV), belonging to 38 species [7 *Alpha*-, 5 *Beta*-, 25 *Gamma*- (18 officially recognized and 6 unclassified), 1 *Mu*-PV], were identified by both PCR systems. Specifically, while CUT PCR identified 67 different HPV types/putative types (14 *Alpha*-, 19 *Beta*- and 33 *Gamma*-PV), 4 of which corresponded to novel putative *Gamma*-PV types (EP06, EP07, EP08 and EP10), *Gamma*-PV PCR identified 56 different HPV types/putative types (15 *Beta*, 40 *Gamma*-, and 1 *Mu*-PV) with 11 of them being potentially novel putative *Gamma*-PV types (EP13, EP15, EP16, EP17, EP18, EP19, EP20, EP21, EP22, EP23 and EP24) (Supp. Table 3). Phylogenetic relationships of representative HPV types and the novel putative HPV types are shown in Supp. Fig. 1A/B.

Both PCRs exhibited similar capacities in detecting viruses from *Gamma*-PV (33 viruses by CUT PCR vs. 40 viruses by *Gamma*-PV PCR) and *Beta*-PV genera (19 viruses by CUT PCR vs. 15 viruses by *Gamma*-PV PCR). Nevertheless, they were able to amplify HPVs from diverse species of both HPV genera, indicating their different specificities as depicted in Fig. 1 and Suppl. Table 3. Particularly, CUT primers appeared to have a higher specificity for *Beta*-1, *Beta*-3, *Gamma*-12 and *Gamma*-Un7 species, while Gamma-PV primers seemed to mostly amplify members of *Beta*-4, *Gamma*-7, *Gamma*-15 and *Gamma*-Un1 species, among others. Despite these observations, both primer systems showed differential capabilities in detecting different HPV types within the same HPV species (Suppl. Table 3). Interestingly, only 11 HPV types were detected by both primer systems (HPV24, HPV36, HPV98, HPV22, HPV110, HPV161, HPV197, HPV-mm090c10, HPV-mw23c77, HPVKN1, HPVSE435), and none of them were novel putative HPV types. On the other hand, *Alpha*- and *Mu*-PV genera were only detected by CUT PCR and *Gamma*-PV PCR, respectively (Supp. Table 3, Fig. 1).

### 3.2. HPV prevalence and genera distribution in healthy skin and cutaneous lesions

As shown in Table 3, HPV DNA was present in 33% of analyzed samples, with most of the detected HPV types/putative types clustering to the *Gamma*-PV genus (51%), followed by *Beta*- (32%) and *Alpha*-PV genera (23%). Regarding the prevalence of specific HPV types/putative types, HPV2 (*Alpha*-4) was the most prevalent HPV type identified in all samples (8%). In addition, mixed infections (infection with more than one HPV type) were detected in 51% of HPV-positive samples.

The prevalence of HPV infection was significantly higher in surface swab samples collected from actinic keratosis (62%) in comparison to swabs collected from other clinical entities, such as benign [seborrhoeic keratosis: 53%, skin warts: 50%, benign skin lesions: 31%] and malignant skin lesions [squamous cell carcinomas: 50%, basal cell carcinoma: 40%], or samples obtained from healthy volunteers (forehead: 40%, eyebrow hairs follicles: 25%) ($p = 0.0014$) (Table 2). In biopsy samples, HPV DNA prevalence was significantly higher in skin warts (36%) and actinic keratosis (33%) with respect to benign (seborrhoeic keratosis: 12% and benign skin lesions: 0%) and malignant (basal cell carcinoma: 16% and squamous cell carcinomas: 11%) skin lesions ($p = 0.0047$) (Table 2).

The analysis of genus-specific HPV DNA prevalence in samples with distinct histopathological diagnoses revealed that *Gamma*-PV types/putative types predominated in all clinical entities and healthy skin, except in biopsies of seborrhoeic keratosis and surface swabs and biopsies of skin warts, in which *Alpha*-PVs were most common

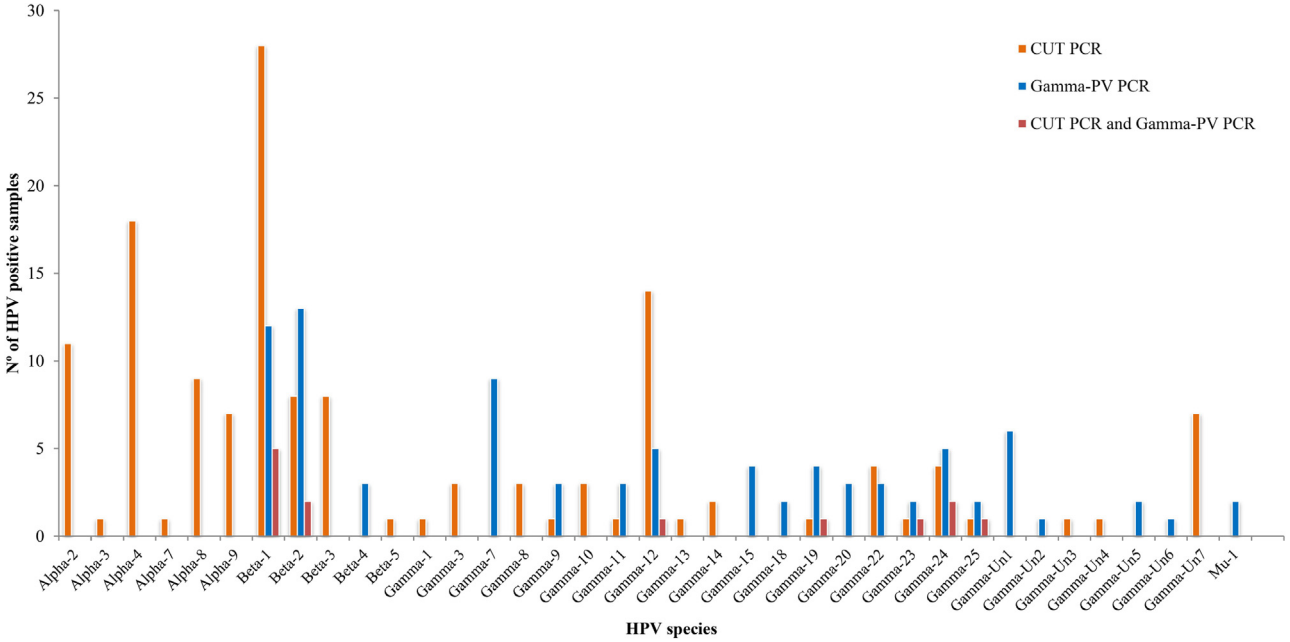


**Fig. 1.** Differential capacity of CUT and Gamma-PV primer systems in detecting HPV types/novel putative types from different genera and species. Un: species not included in the current HPV taxonomy.

**Table 2**
Detection of HPV in 653 samples derived from healthy skin and cutaneous lesions.

| Histopathological diagnosis | Type of sample | HPV positivity *n* (%) | HPV genera *n* (%) | | | | Mixed infections *n* (%) |
|---|---|---|---|---|---|---|---|
| | | | *Alpha*-PV | *Beta*-PV | *Gamma*-PV | *Mu*-PV | |
| Healthy skin | Eyebrow hair follicles (*n* = 77) | 19 (25) | 4 (5) | 3 (4) | 12 (16) | 0 (0) | 9 (12) |
| | Forehead swabs (*n* = 151) | 61 (40) | 16 (11) | 20 (13) | 28 (19) | 0 (0) | 15 (19) |
| Benign skin lesions | Surface swabs (*n* = 13) | 4 (31) | 0 (0) | 0 (0) | 4 (31) | 0 (0) | 2 (15) |
| | Lesion biopsies (*n* = 13) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Seborrhoeic keratosis | Surface swabs (*n* = 17) | 9 (53) | 3 (18) | 3 (18) | 3 (18) | 0 (0) | 4 (24) |
| | Lesion biopsies (*n* = 17) | 2 (12) | 2 (12) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Skin warts | Surface swabs (*n* = 14) | 7 (50) | 5 (36) | 1 (7) | 2 (14) | 0 (0) | 1 (7) |
| | Lesion biopsies (*n* = 14) | 5 (36) | 5 (36) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Actinic keratosis | Surface swabs (*n* = 71) | 44 (62) | 5 (7) | 22 (31) | 24 (34) | 0 (0) | 34 (48) |
| | Lesion biopsies (*n* = 70) | 23 (33) | 2 (3) | 6 (9) | 15 (21) | 0 (0) | 21 (30) |
| Basal cell carcinoma | Surface swabs (*n* = 43) | 17 (40) | 0 (0) | 9 (21) | 10 (23) | 1 (2) | 13 (30) |
| | Lesion biopsies (*n* = 91) | 15 (16) | 5 (5) | 4 (4) | 8 (9) | 1 (1) | 11 (12) |
| Squamous cell carcinoma | Surface swabs (*n* = 8) | 4 (50) | 1 (12.5) | 1 (12.5) | 2 (25) | 0 (0) | 0 (0) |
| | Lesion biopsies (*n* = 54) | 6 (11) | 2 (4) | 1 (2) | 3 (6) | 0 (0) | 1 (2) |
| **Total** | **653** | **216 (33)** | **50 (23)** | **70 (32)** | **111 (51)** | **2 (0)** | **111 (51)** |

**Table 3**
The main genomic features and putative proteins of a novel HPV type, HPV210.

| Putative genomic regions/ ORFs | Length (nt) | Nucleotide sequence (pre-stop codon) (nt) | Protein size (aa) | HPV motifs and domains (consensus sequences) | Genomic position | Nucleotide identities with HPV132 (%) |
|---|---|---|---|---|---|---|
| *URR* | 462 | 6671–7134 | – | Polyadenylation site (AATAAA) | nt 6722–6727<br>nt 6776–6781 | 83 |
| | | | | TATA box (TATAAA) | nt 6817–6822<br>nt 7077–7082<br>nt 7099–7104 | |
| | | | | E1-binding site (ATGATAGTTGCCAACTATC) | nt 7046–7064 | |
| | | | | E2-binding site [ACC(N)$_6$GGT] | nt 6876–6887<br>nt 6916–6927<br>nt 7016–7027<br>nt 7086–7097 | |
| *E6* | 420 | 1–420 | 139 | Zinc-binding domain [CXXC(X)$_{29}$CXXC] | aa 27–63<br>aa 100–136 | 84 |
| | | | | PDZ-binding domain [X(T/S)X(L/V)] | aa 90–93<br>aa 31–34<br>aa 47–50<br>aa 20–23 | |
| *E7* | 279 | 417–695 | 92 | Zinc-binding domain [CXXC(X)$_{29}$CXXC] | aa 45–81 | 94 |
| | | | | pRB-binding site (LXCXE) | aa 22–26 | |
| *E1* | 1800 | 679–2478 | 599 | Bipartite-like NLS [KRK(X)$_{27}$KRRL] | aa 78–111 | 89 |
| | | | | NES [L(X)$_{2-3}$L(X)$_2$(L,I,V)X(L,I)] | aa 92–101 | |
| | | | | ATP-binding site [GXXXXGK(T/S)] | aa 427–434 | |
| | | | | Cdk-phosphorylation site [(S/T)P] | aa 461–462<br>aa 84–85<br>aa 93–94<br>aa 102–103 | |
| | | | | Cyclin-binding motif (RXL) | aa 502–504<br>aa 568–570<br>aa 109–111<br>aa 87–89 | |
| *E2* | 1092 | 2405–3496 | 363 | Leucine zipper domain [L(X)$_6$L(X)$_6$L(X)$_6$L] | absent | 88 |
| *L2* | 1542 | 3593–5134 | 513 | Polyadenylation site (AATAAA) | nt 3686–3691<br>nt 4382–4387<br>nt 4765–4770<br>nt 4972–4977<br>nt 5076–5081 | 82 |
| | | | | Furin cleavage motif [RX(K/R)R] | aa 7–10 | |
| | | | | Transmembrane-like domain [G(X)$_{26}$G] | aa 57–83 | |
| *L1* | 1527 | 5145–6671 | 508 | | | 82 |

cdk, cyclin/cyclin-dependent kinase; NES, nuclear export signal; NLS, nuclear localization signal; pRB, retinoblastoma protein; URR, upstream regulatory region.

(Table 2). Moreover, significantly higher prevalence of *Alpha*-PVs was found in both surface swabs of lesions and biopsies of skin warts with respect to equivalent samples of other skin pathologies or healthy skin samples ($p = 0.0041$ and $p = 0.0042$, respectively). While similar rates of *Gamma*-PV infections were detected in surface swabs of lesions and healthy skin samples ($p = 0.1830$), a higher frequency of *Beta*-PVs was detected in surface swabs of actinic keratosis ($p = 0.0004$). In biopsy samples, a significantly higher prevalence of *Gamma*-PVs was found in actinic keratosis in comparison to other skin pathologies ($p = 0.0172$); however, no differences in *Beta*-PV prevalence were observed between

the mentioned groups of samples ($p = 0.536$).

Mixed infection rates were significantly higher in surface swabs of actinic keratosis (48%) in comparison to surface swab samples obtained from other skin pathologies or healthy skin samples ($p = 0.00001$) (Table 2). The same trend was found in biopsies, with higher rates of mixed infections in actinic keratosis (30%) with respect to basal cell carcinoma, squamous cell carcinomas, seborrhoeic keratosis, skin warts and benign skin lesions ($p = 0.00001$).

Due to the high HPV detection rates in patients with actinic keratosis, we further investigated HPV genus-specific prevalence in their surface swabs and biopsies (Table 2). In surface swabs, the comparative analysis demonstrated that *Beta*- and *Gamma*-PV prevalence were similar (31% and 34%, respectively) and higher than *Alpha*-PV prevalence (7%) ($p = 0.0003$). Interestingly, when analyzing genus-specific prevalence in actinic keratosis biopsies, *Gamma*-PVs' prevalence was significantly higher (21%) than *Beta*- and *Alpha*-PVs (9% and 3%, respectively) ($p = 0.002$). Taking into account that actinic keratosis is the precursor lesion of NMSC, the same analysis was performed in surface swabs and biopsies of squamous and basal cell carcinoma (Table 2). In surface swabs, similar trends to actinic keratosis were found in basal cell carcinoma specimens, with similar and higher frequencies of *Beta*- and *Gamma*-PVs with respect to *Alpha*-PVs ($p = 0.004$), while no particular genera predominated in squamous cell carcinomas ($p = 0.7788$). However, in contrast to actinic keratosis biopsies, no HPV genus prevailed in analyzed biopsy samples of basal cell carcinoma ($p = 0.4204$) and squamous cell carcinomas ($p = 0.6065$).

Upon comparison of HPV infections in swabs and biopsies of each clinical entity (Table 2), significantly higher prevalence of HPV infection was found in surface swabs with respect to biopsies of actinic keratosis ($p = 0.0002$) and seborrhoeic keratosis ($p = 0.0391$). On the other hand, these differences were not found in skin warts ($p = 0.50$), basal cell carcinoma ($p = 0.1088$) and squamous cell carcinomas ($p = 0.50$) sample sets. When comparing swabs and biopsies of the same patient, HPV infection of both samples was detected in a low proportion of subjects [seborrhoeic keratosis 12% (2/17); skin warts 29% (4/14); actinic keratosis 27% (19/70); basal cell carcinoma 16% (7/43); squamous cell carcinomas 25% (2/8)]. Furthermore, the same HPV type was simultaneously identified in a very few pairs of samples [seborrhoeic keratosis 12% (2/17); skin warts 21% (3/14); actinic keratosis 6% (4/70); basal cell carcinoma 5% (2/43); squamous cell carcinomas 12.5% (1/8)] (data not shown).

### 3.3. Characterization of HPV210, a novel HPV type clustering to species *Gamma-12*

As shown in Fig. 2, phylogenetic analysis and pairwise comparison of L1 ORFs confirmed that HPV210 is a novel member of the *Gamma*-PV genus, with nucleotide identities with viruses clustering within different *Gamma*-PV species ranging from 66% to 82% (data not shown). The novel HPV type represents the seventh officially recognized member of the species *Gamma*-12, showing the highest L1 ORF identity to HPV132 (82%).

The full genome sequence of the novel HPV type, HPV210, was determined to be 7134-bp in length with a GC content of 38%. The *in silico* analysis of the HPV210 viral genome, depicted in Table 3, showed the typical genomic organization of cutaneous HPVs, potentially encoding 4 early (E6, E7, E1 and E2) and 2 late genes (L2 and L1), as well as missing the E5 ORF, which is a characteristic feature of human *Beta*-, *Gamma*-, *Mu*- and *Nu*-PVs (Chouhy et al., 2013a).

No canonical E4 ORF nested within the E2 could be identified in the genome of HPV210. However, a short sequence of 121 amino acids in length (nt position 2986-3351), with residues of the proline-rich stretches that characterize E4, was detected. Although no start codons were found in this sequence, the putative E4 protein of HPV210 is likely to be translated from a spliced mRNA, consisting of the first few codons of the E1 ORF joined to the E4 ORF (the E1^E4 spliced mRNA), as shown for other HPVs (Doorbar, 2013; Kovanda et al., 2011). The putative HPV210 E4 protein showed the highest similarity (89%) to the HPV132 E4 protein.

Within the upstream regulatory region (URR) of HPV210, 3 typical TATA boxes, 4 putative binding sites for the E2 protein, a binding site for the E1 protein (Oštrbenk et al., 2015), and 2 putative polyadenilation sites for late gene transcripts were identified. Multiple potential binding sites for transcriptional regulatory factors, such as AP-1, NF-1 and SP-1, were also present within its URR (data not shown).

The E6 ORF contained 2 characteristic zinc-binding domains, separated by 36 amino acids (Ullman et al., 1996), and 4 internal and likely not functional PDZ-binding motifs (QTPL, FTDV, KSWL, LSLL) (Fanning and Anderson, 1999). The E7 ORF contained a pRB-binding motif (LYCYD) (Radulescu et al., 1995) and a single zinc-binding domain. Analysis of the putative E1 gene product, the largest protein encoded by HPV210, showed a typical ATP-binding site of the ATP-dependent helicase (GPSDTGKS) (Titolo et al., 1999), and several Cdk-phosphorylation and Cyclin-binding sites. At the N-termini of the E1 protein, a highly conserved bipartite-like nuclear localization signal (NLS) and a leucine-rich Crm1-dependent nuclear export signal (NES) (LSPRLQAVHI), which together enable shuttling of the E1 protein between the cell nucleus and cytoplasm in most HPVs, were identified (Mantovani and Banks, 2001; Lange et al., 2007; Fradet-Turcotte et al., 2010). No conserved leucine zipper domain was present at the C-termini of the putative HPV210 E2 protein, in agreement with other members of the species *Gamma*-12 (Oštrbenk et al., 2015). At the N-termini of the putative L2 protein, a highly conserved furin cleavage motif (RTRR), as well as a transmembrane-like domain (GIGTGRGSGGSLGYRPLGEGGSGRPIG) (Wang and Roden, 2013), were identified. In addition, five canonical polyadenylation sites, necessary for regulation of early viral transcripts (Johansson and Schwartz, 2013), were also found in HPV210 L2, as shown in Table 3.

## 4. Discussion

In recent years, sensitive conventional molecular detection methods (Chouhy et al., 2013a, 2018; Bolatti et al., 2017) and NGS (Foulonge et al., 2012, Ekström et al., 2013; Bzhalava et al., 2014; Brancaccio and Robitaille et al., 2018) have allowed the identification of a considerable number of novel HPV types. Nonetheless, using NGS technology, Arroyo Mühr and colleagues have demonstrated that the HPV types/putative types can be detected with higher sensitivity when sequencing PCR amplicons with respect to sequencing only extracted DNA, without prior PCR amplification, in aliquots of the same samples (Arroyo Mühr et al., 2015). While metagenomics is a powerful approach, the application of techniques with higher sensitivity is still required when studying low-level HPV persistence, particularly in skin infections. In this work, we developed the *Gamma*-PV PCR that mostly amplifies HPV types from the *Gamma*-PV genus, the largest and most diverse PV genus characterized so far. When comparing the capacity of the novel *Gamma*-PV PCR in detecting and differentiating distinct HPV infections to the well-known CUT PCR (Chouhy et al., 2010; Brancaccio and Robitaille et al., 2018), based on the analysis of 653 samples originating from the cutaneous epithelium, both PCR protocols showed different specificities and, if used in a combination, covered almost the complete diverse spectrum of HPVs, enabling the amplification of most HPV types belonging to 4 out of 5 HPV genera. Although there was no apparent correlation between HPV detection and the number of mismatches at the corresponding binding regions of each primer system, it should be considered that the strategies used for designing *Gamma*-PV and CUT primers were different. Specifically, *Gamma*-PV PCR was designed to amplify *Gamma*-PV types and included 2 primers with conserved sequences at their 3′ regions, while the CUT PCR includes 5 pan-generic primers designed to detect HPVs from all five genera (Chouhy et al., 2010).

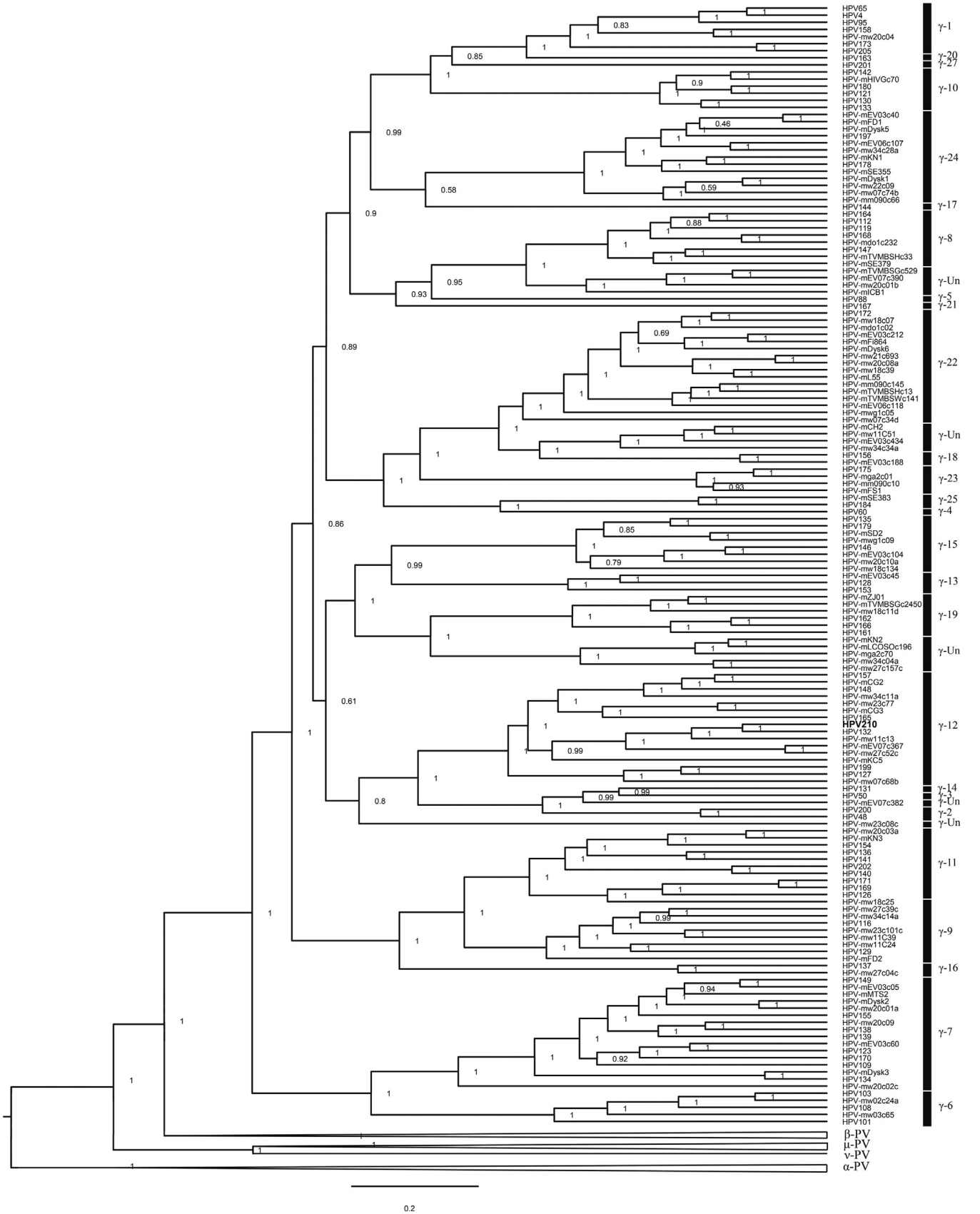


**Fig. 2.** Phylogenetic position of HPV210 with respect to reference HPVs, obtained with the phylogenetic analysis of L1 ORF sequences of the novel HPV type characterized in this study and 286 reference HPVs from *Alpha*-, *Beta*-, *Gamma*-, *Mu*- and *Nu*-PV genera. Only bayesian posterior probability values (BPP) of > 0.50 are shown. Novel HPV210 type is shown in bold. *Alpha*-, *Beta*-, *Mu*- and *Nu*-PV genera branches are collapsed. The raw phylogenetic trees are available upon request.

It should be noted that although international consensus for HPV classification is based on the L1 ORF (de Villiers et al., 2004), we applied the same criterion for the identification of novel putative HPV types amplified with the *Gamma*-PV PCR (E1 ORF). Even though we are aware about the reported differences in the relative divergences (García-Vallvé et al., 2005) and different topologies of phylogenetic trees of early and late HPV genes (Bravo and Alonso, 2007), similar nucleotide identities among L1 and E1 ORFs were obtained in the pairwise comparison analysis of HPV types from *Alpha*-, *Beta*-, *Gamma*- and *Mu*-PV genera, respectively (Supp. Table 2; *Alpha*-PV: 3%, *Beta*-PV: −0,06%, *Gamma*-PV: −1%, *Mu*-PV: −0.7%). In addition, phylogenetic trees of novel putative HPV types, based on L1 or E1 nucleotide sequences, have resolved properly with high posterior probability values, and all "EP types" clustered into HPV species according to their nucleotide identities. However, as most putative HPV types have been detected using general primers targeting the L1 ORF (Chouhy et al., 2013b), we cannot ensure that the 11 novel putative HPV types identified in our study, using the *Gamma*-PV PCR, are indeed novel putative HPV types, as they could also represent previously described putative HPV types.

Several studies have suggested that *Beta*-PV types contribute to actinic keratosis and NMSC development, acting as co-factors next to UV-radiation (Feltkamp, 2008; Quint et al., 2015). Nevertheless, the majority of epidemiological studies focused their efforts only on finding *Beta*-PV types in pre-malignant and malignant skin lesions (Bouwes Bavinck et al., 2010; Proby et al., 2011; Borgogna et al., 2014; Zakrzewska et al., 2012). Moreover, these studies were additionally biased to detecting only some specific *Beta*-PV types (Cubie, 2013; Harwood et al., 1999) and did not target other HPV genera that have also been associated with skin pathologies (Meyers et al., 2018). Consequently, *Gamma*-PVs have been poorly studied in the association with the development of malignant lesions and only scarce published data are available on their role in the development of NMSC (Grace and Munger, 2017; Meyers et al., 2018). Previous studies have shown that *Gamma*-PVs can be etiologically linked to sporadic cases of common warts, especially in immunosuppressed patients (Hošnjak et al., 2015; Cubie, 2013; Harwood et al., 1999; Köhler et al., 2011). In addition, *Gamma*-PVs have also been identified in squamous cell carcinomas (Arroyo Mühr et al., 2015; Ekström et al., 2013) and actinic keratosis (Ekström et al., 2013; Köhler et al., 2009).

To expand the knowledge on the etiological association of HPVs, especially *Gamma*-PVs, with cutaneous neoplasms, we assessed the HPV prevalence and type distribution in 653 samples derived from 448 immunocompetent individuals. Through the comparison of biopsies derived from individuals with benign (seborrhoeic keratosis, skin warts and benign skin lesions), pre-malignant (actinic keratosis) and malignant (basal and squamous cell carcinomas) skin lesions, the highest HPV DNA prevalence was observed in actinic keratosis (33%) and skin warts (36%) tissue specimens ($p = 0.0047$), while the highest rate of multiple infections was found in actinic keratosis (30%) ($p = 0.00001$). Our results are consistent with other studies reporting higher HPV prevalence and viral loads in actinic keratosis compared to squamous cell carcinomas biopsies (Pfister et al., 2003; Weissenborn et al., 2005).

It has been reported that eyebrow hairs follicles (Schneider et al., 2013) and surface swabs (Alotaibi et al., 2006) from actinic keratosis patients harbor higher numbers of cutaneous HPVs in comparison with healthy individuals. Similar trends were also observed in our study, with higher prevalence of overall HPV infection and mixed infections in surface swabs of actinic keratosis lesions in comparison with swabs of other pathologies and healthy skin (forehead and eyebrow hairs samples) ($p = 0.0014$ and $p = 0.00001$, respectively).

Previous findings indicated that HPV DNA is common in superficial layers of lesions but is not necessarily present throughout whole thickness of biopsy tissues (Forslund et al., 2004). Interestingly, in comparison with other benign and malignant skin lesions, the highest prevalence of *Gamma*-PV infection was found in actinic keratosis biopsies (21%) ($p = 0.0172$), while the *Gamma*-PV prevalence in swabs obtained from the surface of cutaneous lesions and healthy skin was similar ($p = 0.1830$). On the other hand, *Beta*-PVs were more prevalent in surface swabs of actinic keratosis with respect to comparable samples from other pathologies and healthy skin ($p = 0.0004$). However, similarly low *Beta*-PV prevalence was found in all biopsies ($p = 0.536$).

Our results suggest a potential active role of *Gamma*-PVs in the development of pre-malignant cutaneous lesions. Namely, the comparison of HPV genera-specific prevalence demonstrated higher rates of *Gamma*-PVs in actinic keratosis biopsies (21%) compared to *Beta*- (9%) and *Alpha*-PVs (3%) ($p = 0.002$). Current evidence suggests that cutaneous HPVs could have adopted a "hit-and-run" mechanism in the development of cutaneous neoplasms, in which viral oncogene expression plays a role in the initiation of cell transformation but is ultimately no longer required for tumor maintenance (Aldabagh et al., 2013; Hasche et al., 2017; Meyers et al., 2018). In agreement with this observation, on the contrary to actinic keratosis biopsies, lower rates of overall HPV infection and multiple infections were observed in both basal and squamous cell carcinomas, supporting the idea that viral DNA is lost during progression from actinic keratosis to NMSC (Weissenborn et al., 2005).

Additionally, we fully characterized the genome sequence of HPV210, a novel HPV type clustering to the *Gamma*-12 species, from a swab sample of healthy skin. Even though further studies are needed to determine the clinical relevance of the novel virus, the presence of a pRB-binding motif in the E7 ORF might indicate that the novel *Gamma*-PV type has transforming properties. Interestingly, it has been suggested recently that the pRB-binding motif has been lost during the evolutionary history of the species *Gamma*-12, leading to some HPV types lacking the conserved LxCxE motif (Oštrbenk et al., 2015). In accordance with these findings, HPV210 is phylogenetically-related to HPV types with a conserved LxCxE motif (HPV132 and HPV-mw11c13). Notably, HPV148, containing a pRB-binding motif, was detected in actinic keratosis biopsy of an immunosuppressed patient, provoking more questions about the potential role of *Gamma*-PVs in the malignant transformation of the skin (Köhler et al., 2011).

In conclusion, a newly designed sensitive PCR protocol for amplification of *Gamma*-PV types can be considered as an additional tool in epidemiological studies directed to better understand the clinical implications of *Gamma*-PV infections. The high prevalence of HPV DNA infections, mixed infections and *Gamma*-PV types in biopsy samples of actinic keratosis supports a possible early role of *Gamma*-PVs in the development of malignant cutaneous lesions; however, the pathogenicity of these HPV types should be studied in greater details. The characterization of HPV210, the seventh officially recognized member of the species *Gamma*-12, with a currently unknown clinical significance, expands the current knowledge of PV diversity.

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## Conflict of interest

The authors declare that they have no competing interests.

## Ethical statement

The study was conducted according to the Declaration of Helsinki and was approved by the Institutional Review Board of the Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Rosario, Argentina (reference number 6060/134). All subjects provided written informed consents for participation in the study.

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.virol.2018.09.006

## References

Akgül, B., Cooke, J.C., Storey, A., 2006. HPV-associated skin disease. J. Pathol. 208, 165–175.
Alotaibi, L., Provost, N., Gagnona, S., Franco, E.L., Coutlee, F., 2006. Diversity of cutaneous human papillomavirus types in individuals with and without skin lesión. J. Clin. Virol. 36, 133–140.
Aldabagh, B., Angeles, J.G., Cardones, A.R., Arron, S.T., 2013. Cutaneous squamous cell carcinoma and human papillomavirus: is there an association? Dermatol. Surg. 9, 1–23.
Arroyo Mühr, L.S., Hultin, E., Bzhalava, D., Eklund, C., Lagheden, C., et al., 2015. Human papillomavirus type 197 is commonly present in skin tumors. Int. J. Cancer 136, 2546–2555.
Bolatti, E.M., Chouhy, D., Casal, P.E., Perez, G.R., Stella, E.J., et al., 2016. Characterization of novel human papillomavirus types 157, 158 and 205 from healthy skin and recombination analysis in genus Gamma-Papillomavirus. Infect. Genet. Evol. 42, 20–29.
Bolatti, E.M., Chouhy, D., Hošnjak, L., Casal, P.E., Kocjan, B.J., et al., 2017. Natural history of human papillomavirus infection of sunexposed healthy skin of immunocompetent individuals over three climatic seasons and identification of HPV209, a novel betapapillomavirus. J. Gen. Virol. 98, 1334–1348.
Borgogna, C., Lanfredini, S., Peretti, A., De Andrea, M., Zavattaro, E., 2014. Improved detection reveals active beta-papillomavirus infection in skin lesions from kidney transplant recipients. Mod. Pathol. 27, 1101–1115.
Bouwes Bavinck, J.N., Neale, R.E., Abeni, D., Euvrard, S., Green, A.C., et al., 2010. Multicenter study of the association between betapapillomavirus infection and cutaneous squamous cell carcinoma. Cancer Res. 70, 9777–9786.
Brancaccio, R.N., Robitaille, A., Duttaa, S., Cuenina, C., Santareb, D., Skendersb, G., Lejab, M., Fischerc, Giulianoe, A.R., Rollisone, D.E., Grundhoffd, A., Tommasino, M., Gheita, T., 2018. Generation of a novel next-generation sequencing-based method for the isolation of new human papillomavirus types. Virology 520, 1–10.
Bravo, I.G., Alonso, A., 2007. Phylogeny and evolution of papillomaviruses based on the E1 and E2 proteins. Virus Genes 34, 249–262.
Bravo, I.G., de Sanjose, S., Gottschling, M., 2010. The clinical importance of understanding the evolution of papillomaviruses. Trends Microbiol. 18, 432–438.
Bzhalava, D., Johansson, H., Ekström, J., Faust, H., Möller, B., et al., 2013. Unbiased approach for virus detection in skin lesions. PLoS One 8, e65953.
Bzhalava, D., Arroyo Mühr, L.S., Lagheden, C., Ekström, J., Forslund, O., et al., 2014. Deep sequencing extends the diversity of human papillomaviruses in human skin. Sci. Rep. 4, 5807. https://doi.org/10.1038/srep05807.
Chahoud, J., Semaan, A., Chen, Y., Cao, M., Rieber, A.G., et al., 2016. Association between b-genus human papillomavirus and cutaneous squamous cell carcinoma in immunocompetent Individuals—a metaanalysis. JAMA Dermatol. 152, 1354–1364.
Chouhy, D., Gorosito, M., Sanchez, A., Serra, E.C., Bergero, A., et al., 2010. New generic primer system targeting mucosal/genital and cutaneous human papillomaviruses leads to the characterization of HPV 115, a novel Beta-papillomavirus species 3. Virology 397, 205–216.
Chouhy, D., Bolatti, E.M., Piccirilli, G., Sanchez, A., Fernandez Bussy, R., et al., 2013a. Identification of human papillomavirus type 156, the prototype of a new human gammapapillomavirus species, by a generic and highly sensitive PCR strategy for long DNA fragments. J. Gen. Virol. 94, 524–533.
Chouhy, D., Bolatti, E.M., Perez, G.R., Giri, A.A., 2013b. Analysis of the genetic diversity and phylogenetic relationships of putative human papillomavirus types. J. Gen. Virol. 94, 2480–2488.
Chouhy, D., Kocjan, B.J., Staheli, J.P., Bolatti, E.M., Hošnjak, L., et al., 2018. Detection of novel Betapapillomaviruses and Gammapapillomaviruses in eyebrow hair follicles using a single-tube 'hanging droplet' PCR assay with modified Pan-PV CODEHOP primers. J. Gen. Virol. 99, 109–118.
Cubie, H.A., 2013. Diseases associated with human papillomavirus infection. Virology 445, 21–34.
de Villiers, E.M., Fauquet, C., Broker, T.R., Bernard, H.U., Zur Hausen, H., 2004. Classification of papillomaviruses. Virology 324, 17–27.
de Villiers, E.M., 2013. Cross-roads in the classification of papillomaviruses. Virology 445, 2–10.
Doorbar, J., 2013. The E4 protein; structure, function and patterns of expression. Virology 445, 80–98.
Drummond, A.J., Suchard, M.A., Xie, D., Rambaut, A., 2012. Bayesian phylogenetics with BEAUti and the BEAST 1.7. Mol. Biol. Evol. 29, 1969–1973.
Ekström, J., Arroyo Mühr, L.S., Bzhalava, D., Söderlund-Strand, A., Hultin, E., et al., 2013. Diversity of human papillomaviruses in skin lesions. Virology 447, 300–311.
Fanning, A.S., Anderson, J.M., 1999. PDZ domains: fundamental building blocks in the organization of protein complexes at the plasma membrane. J. Clin. Investig. 103, 767–772.
Feltkamp, M.C., de Koning, M.N., Bavinck, J.N., Ter Schegget, J., 2008. Betapapillomaviruses: innocent bystanders or causes of skin cancer. J. Clin. Virol. 43, 353–360.
Forslund, O., Antonsson, A., Nordin, P., Stenquist, B., Hansson, B.G., 1999. A broad range of human papillomavirus types detected with a general PCR method suitable for analysis of cutaneous tumours and normal skin. J. Gen. Virol. 80, 2437–2443.
Forslund, O., Lindelof, B., Hradil, E., Nordin, P., Stenquist, B., et al., 2004. High prevalence of cutaneous human papillomavirus DNA on the top of skin tumors but not in “Stripped” biopsies from the same tumors. J. Investig. Dermatol. 123, 388–394.
Fradet-Turcotte, A., Moody, C., Laimins, L.A., Archambault, J., 2010. Nuclear export of human papillomavirus type 31 E1 is regulated by Cdk2 phosphorylation and required for viral genome maintenance. J. Virol. 84, 11747–11760.
García-Vallvé, S., Alonso, A., Bravo, I.G., 2005. Papillomaviruses: different genes have different histories. Trends Microbiol. 13, 514–521.
Grace, M., Munger, K., 2017. Proteomic analysis of the gamma human papillomavirus type 197 E6 and E7 associated cellular proteins. Virology 500, 71–81.
Hasche, D., Stephan, S., Braspenning-Wesch, I., Mikulec, J., Niebler, M., et al., 2017. The interplay of UV and cutaneous papillomavirus infection in skin cáncer development. PLoS Pathog. 13, e1006723.
Harwood, C.A., Spink, P.J., Surentheran, T., Leigh, I.M., de Villiers, E.M., McGregor, J.M., et al., 1999. Degenerate and nested PCR: a highly sensitive and specific method for detection of human papillomavirus infection in cutaneous warts. J. Clin. Microbiol. 37, 3545–3555.
Hošnjak, L., Kocjan, B.J., Pirš, B., Seme, K., Poljak, M., 2015. Characterization of two novel gammapapillomaviruses, HPV179 and HPV184, isolated from common warts of a renal-transplant recipient. PLoS One 10 (e0119154).
Howley, P.M., Pfister, H.J., 2015. *Beta* genus papillomaviruses and skin cancer. Virology 479–480, 290–296.
Johansson, C., Schwartz, S., 2013. Regulation of human papillomavirus gene expression by splicing and polyadenylation. Nat. Rev. Microbiol. 11, 239–251.
Kalendar, R., Lee, D., Schulman, A.H., 2014. FastPCR software for PCR, in silico PCR, and oligonucleotide assembly and analysis. Methods Mol. Biol. 1116, 271–302.
Katoh, Rozewicki, Yamada, 2017. MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. Brief. Bioinforma. 1–7. https://doi.org/10.1093/bib/bbx108.
Kocjan, B.J., Poljak, M., Seme, K., Potocnik, M., Fujs, K., Babic, D.Z., 2005. Distribution of human papillomavirus genotypes in plucked eyebrow hairs from Slovenian males with genital warts. Infect. Genet. Evol. 5, 255–259.
Köhler, A., Meyer, T., Stockfleth, E., Nindl, I., 2009. High viral load of human wart-associated papillomaviruses (PV) but not beta-PV in cutaneous warts independent of immunosuppression. Br. J. Dermatol. 161, 528–535.
Köhler, A., Gottschling, M., Manning, K., Lehmann, M., Schulz, E., et al., 2011. Genomic characterization of ten novel cutaneous human papillomaviruses from keratotic lesions of immunosuppressed patients. J. Gen. Virol. 92, 1585–1594.
Kovanda, A., Kocjan, B.J., Potocnik, M., Poljak, M., 2011. Characterization of a novel cutaneous human papillomavirus genotype HPV-125. PLoS One 6, e22414.
Lange, A., Mills, R.E., Lange, C.J., Stewart, M., Devine, S.E., et al., 2007. Classical nuclear localization signals: definition, function, and interaction with importin alpha. J. Biol. Chem. 282, 5101–5105.
Lazarczyk, M., Cassonnet, P., Pons, C., et al., 2009. The EVER proteins as a natural barrier against papillomaviruses: a new insight into the pathogenesis of human papillomavirus infections. Microbiol. Mol. Biol. Rev. 73, 348–370.
Mantovani, F., Banks, L., 2001. The human papillomavirus E6 protein and its contribution to malignant progression. Oncogene 20, 7874–7887.
McLaughlin-Drubin, M.E., 2015. Human papillomaviruses and non-melanoma skin cancer. Semin. Oncol. 42, 284–290.
Meyers, J.M., Grace, M., Uberoi, A., Lambert, P.F., Munger, K., 2018. Inhibition of TGF-*beta* and NOTCH signaling by cutaneous papillomaviruses. Front. Microbiol. 9, 389 (Doi 10.3389-fmicb.2018.00389).
Nindl, I., Gottschling, M., Stockfleth, E., 2007. Human papillomavirusesand non-melanoma skin cancer: basic virology and clinical manifestations. Dis. Markers 23, 247–259.
Oštrbenk, A., Kocjan, B.J., Hošnjak, L., Li, J., Deng, Q., et al., 2015. Identification of a

novel human papillomavirus, typeHPV199, isolated from a nasopharynx and anal canal, and complete genomic characterization of papillomavirus species Gamma-12. PLoS One 10 (e0138628).
Pfister, H., 2003. Chapter 8: human papillomavirus and skin cancer. J. Natl. Cancer Inst. Monogr. 31, 52–56.
Pfister, H., Fuchs, P.G., Majewski, S., Jablonska, S., Pniewska, I., Malejczyk, M., 2003. High prevalence of epidermodysplasia verruciformis-associated human papillomavirus DNA in actinic keratosis of the immunocompetent population. Arch. Dermatol. Res. 295, 273–279.
Poljak, M., Kocjan, B.J., Oštrbenk, A., Seme, K., 2016. Commercially available molecular tests for human papillomaviruses (HPV): 2015 update. J. Clin. Virol. 76 (Suppl. 1), S3–S13.
Proby, C.M., Harwood, C.A., Neale, R.E., et al., 2011. A case– control study of betapapillomavirus infection and cutaneous squamous cell carcinoma in organ transplant recipients. Am. J. Transplant. 11, 1498–1508.
Quint, K.D., Genders, R.E., de Koning, M.N., Borgogna, C., Gariglio, M., et al., 2015. Human Beta-papillomavirus infection and keratinocyte carcinomas. J. Pathol. 235, 342–354.
Radulescu, R.T., Bellitti, M.R., Ruvo, M., Cassani, G., Fassina, G., 1995. Binding of the LXCXE insulin motif to a hexapeptide derived from retinoblastoma protein. Biochem. Biophys. Res. Commun. 206, 97–102.
Saiki, R.K., Bugawan, T.L., Horn, G.T., Mullis, K.B., Erlich, H.A., 1986. Analysis of enzymatically amplified beta-globin and HLA-DQ alpha DNA with allele-specific oligonucleotide probes. Nature 324, 163–166.
Schneider, I., Lehmann, M.D., Kogosov, V., Stockfleth, E., Nindl, I., 2013. Eyebrow hairs from actinic keratosis patients harbor the highest number of cutaneous human papillomaviruses. BMC Infect. Dis. 13, 186. https://doi.org/10.1186/1471-2334-13-186.
Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. Mol. Biol. Evol. 30, 2725–2729.
Titolo, S., Pelletier, A., Sauve, F., Brault, K., Wardrop, E., et al., 1999. Role of the ATP-binding domain of the human papillomavirus type 11 E1 helicase in E2-dependent binding to the origin. J. Virol. 73, 5282–5293.
Ullman, C.G., Haris, P.I., Galloway, D.A., Emery, V.C., Perkins, S.J., 1996. Predicted alpha-helix/b-sheet secondary structures for the zinc-binding motifs of human papillomavirus E7 and E6 proteins by consensus prediction averaging and spectroscopic studies of E7. Biochem. J. 319, 229–239.
Van Doorslaer, K., Li, Z., Xirasagar, S., Maes, P., Kaminsky, D., et al., 2017. The Papillomavirus Episteme: a major update to the papillomavirus sequence database. Nucleic Acids Res. 45, 499–506.
Wang, J.W., Roden, R.B., 2013. L2, the minor capsid protein of papillomavirus. Virology 445, 175–186.
Weissenborn, S.J., Nindl, I., Purdie, K., Harwood, C., Proby, C., et al., 2005. Human Papillomavirus-DNA Loads in Actinic Keratosis Exceed those in Non-Melanoma Skin Cancers. J. Investig. Dermatol. 125, 93–97.
WHO/ICO, 2010. Information Centre on HPV and Cervical Cancer (HPV Information Centre). Human Papillomavirus and Related Cancers in World. Summary Report.
Zakrzewska, K., Regalbuto, E., Pierucci, F., Arvia, R., Mazzoli, S., et al., 2012. Pattern of HPV infection in basal cell carcinoma and in perilesional skin biopsies from immunocompetent patients. Virol. J. (9), 309.