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Natural history of human papillomavirus infection of sun-exposed healthy skin of immunocompetent individuals over three climatic seasons and identification of HPV209, a novel betapapillomavirus

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Abstract

We present the first longitudinal study reporting the natural history of human papillomavirus (HPV) infection in sun-exposed skin of healthy individuals living in a geographical area in which solar UV radiation is influenced by the ozone content of the atmosphere. During three climatic seasons, skin swab samples were obtained from 78 healthy individuals and the prevalence of cutaneous HPVs was assessed with broad-spectrum FAP and CUT primers and determined at 54, 45 and 47 % in spring, summer and winter, respectively. Frequencies of mixed HPV infections were significantly higher in spring with respect to summer and winter (P=0.02). Seventy-one different HPV types/putative types were identified. While 62 volunteers were HPV-infected in at least one season, 23 had persistent infections. β-PVs ($\beta-1$) were the most prevalent and persistent. Age was associated with both the infection status (P=0.01) and the type of HPV infection (no infection, indeterminate/transient, persistent P=0.02). The molecular/phylogenetic analysis of the newly identified β-PV, officially designated as HPV209, showed that the virus has a typical genomic organization of cutaneous HPVs with five early (E6, E7, E1, E2 and E4) and two late genes (L2 and L1), which clusters to the species $\beta-2$. This provides useful data on cutaneous HPV infections in high UV-exposed regions.

INTRODUCTION

Papillomaviruses (PVs) are small non-enveloped viruses with circular double-stranded DNA genomes of approximately 8 kbp. PVs belong to the family *Papillomaviridae* [1] and are mainly host-specific, infecting more than 20 different mammals, birds and reptiles [2].

By convention, a novel PV type shows less than 90 % nucleotide identity with the L1 gene sequence of any other officially recognized PV type [1, 3]. Presently, over 300 PVs have been identified and most of them were found to be infecting skin and/or mucosa of humans [4].

All human PV (HPV) types are grouped within five genera (α-PV, β-PV, γ-PV, μ-PV and ν-PV) according to the phylogenetic relationships of their complete L1 gene sequences. While mucosal/genital HPV types are mostly grouped into the α-PV genus, cutaneous HPVs are highly divergent, distributed into all five HPV genera and represent approximately 75 % of HPVs described to date [1].

Recent progress in novel molecular diagnostic techniques has greatly expanded the number of HPVs classified as β- or γ-PVs, with more than 100 HPV types clustering to both PV genera [5–7]. Furthermore, DNA sequencing of PCR amplicons, obtained with broad-spectrum primers, has

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Abbreviations: HPV, human papillomavirus; MCMC, Markov-chain Monte Carlo; NES, nuclear export signal; NLS, nuclear localization signal; NMSC, non-melanoma skin cancer; PV, papillomavirus; SPF, sun protection factor; URR, upstream regulatory region.

The GenBank/EMBL/DDBJ accession numbers for the novel HPV type/putative types reported in this paper are EP04 (KY242581), EP05 (KY242582), and HPV209 (KY242583).

One supplementary figure is available with the online Supplementary Material.

identified more than 200 additional putative HPV types (FA, GC, SE, etc.) that mainly cluster to β- and γ-PVs [8].

The majority of cutaneous HPV infections are subclinical and are common in healthy human skin [9, 10]. Although many studies have suggested a possible role of certain β- and γ-PV types in the development of non-melanoma skin cancer (NMSC) in both immunocompetent and immunosuppressed individuals [11–14], their involvement in human carcinogenesis is still unclear. However, it is well known that UV exposure is the major environmental risk factor for the development of NMSC [15]. Cutaneous HPVs are more commonly detected in sun-exposed healthy skin with respect to unexposed areas [16, 17], suggesting a link between HPV and UV radiation [18, 19]. Promoters of some specific β-PV types, such as HPV5 and HPV8, have been shown to be activated by UV radiation [20], leading to the inhibition of DNA repair mechanisms after long periods of UV exposure [18]. Therefore, in comparison to transient HPV infections, persistent infections with cutaneous HPV types may have a higher potential to promote malignant transformation of cells [19, 21]. In contrast to mucosal HPV types, knowledge about the persistence of cutaneous HPV infections is limited; viral infections probably occur very early in life [9, 22] and persist in healthy skin for months [23] or even years [24].

In the last few decades, the progressive depletion of the stratospheric ozone layer has caused concern due to an eventual increase in UV-radiation levels reaching the Earth's surface [25]. Particularly, regions placed very near the Antarctic continent are frequently affected by the 'ozone hole' which develops every year in the springtime [26].

To the best of our knowledge, this is the first longitudinal study reporting the natural history of HPV infection in sun-exposed skin of healthy individuals living in a geographical area in which solar UV radiation is influenced by the ozone content of the atmosphere. Over three climatic seasons, during a 10 month period, skin swab samples obtained from a cohort of immunocompetent individuals with known demographic characteristics and sun-exposure habits, living in Rosario city (Argentina), were tested for the presence of cutaneous HPV types. Additionally, we report the complete molecular and phylogenetic characterization of HPV209, a newly identified HPV type clustering to β-PV species 2, and the identification of two novel putative γ-PV types, EP04 and EP05.

RESULTS

Participants, samples and HPV detection

In total, 114 individuals agreed to participate in the 10 month follow-up study. The flowchart of participants and samples included in the analysis is summarized in Fig. 1. A total of 78 participants (23 male and 55 female) with a median age of 39 years (range, 23–63 years) completed the study protocol. This group of individuals reported a mean time of sun-exposure of 5 h/week (range, 0–25 h/week) and

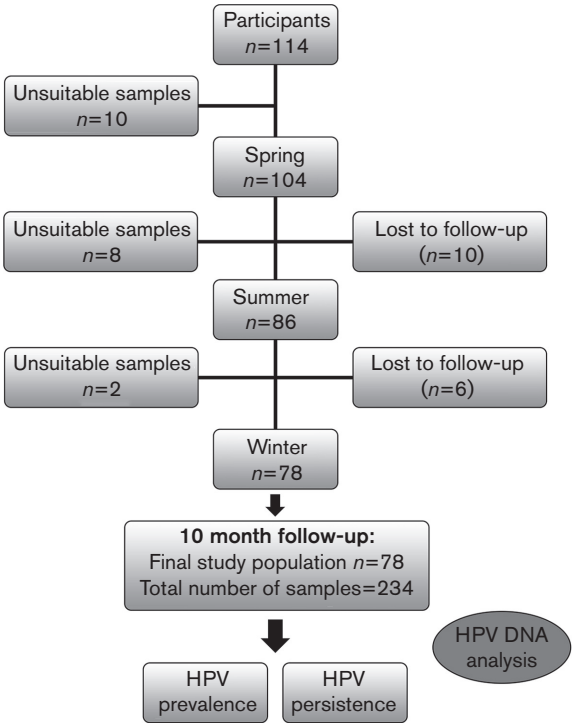

Fig. 1. Flowchart of participant recruitment in three climatic seasons.

all of them were classified as either having skin phototype II (71 %; 55/78) or III (29 %; 23/78). Most participants (58/78) reported that they had been routinely using sunscreen in spring and summer, with the following degrees of protection: 14 % (11/78) low (8–15 SPF); 50 % (39/78) medium (20–30 SPF); and 10 % (8/78) high (35–50 SPF).

Only β-globin-positive samples were considered for downstream HPV analyses. In total, 234 skin samples were considered for analyses of HPV prevalence and persistence (Fig. 1). Overall, HPV DNA was present in 49 % (114/234) of all tested samples. In comparison to the CUT primer system, HPV DNA was more often detected when using FAP primers (22/234 versus 107/234). In addition, 15 and 120 samples were HPV DNA-positive and HPV DNA-negative, respectively, using both primer systems leading to a general concordance of 57.7 % (135/234) [κ=0.0907; IC95 %: 0.0086-0.1729].

Fig. 2 shows phylogenetic relationships of HPV types/putative types identified in skin samples collected during the three climatic seasons. Overall, 71 different HPV types/putative types (α-PVs, 40 β-PVs and 25 γ-PVs) clustered into 21 species, but only six HPV types/putative types were detected by both primer systems. FAP primers identified 56 different HPV types/putative types clustering into 14 species (four β-PV and 10 γ-PV), while CUT primers detected 21 HPV types/putative types belonging to 15 different species (four α-PV, two β-PV and nine γ-PV), two of which corresponded to the only novel

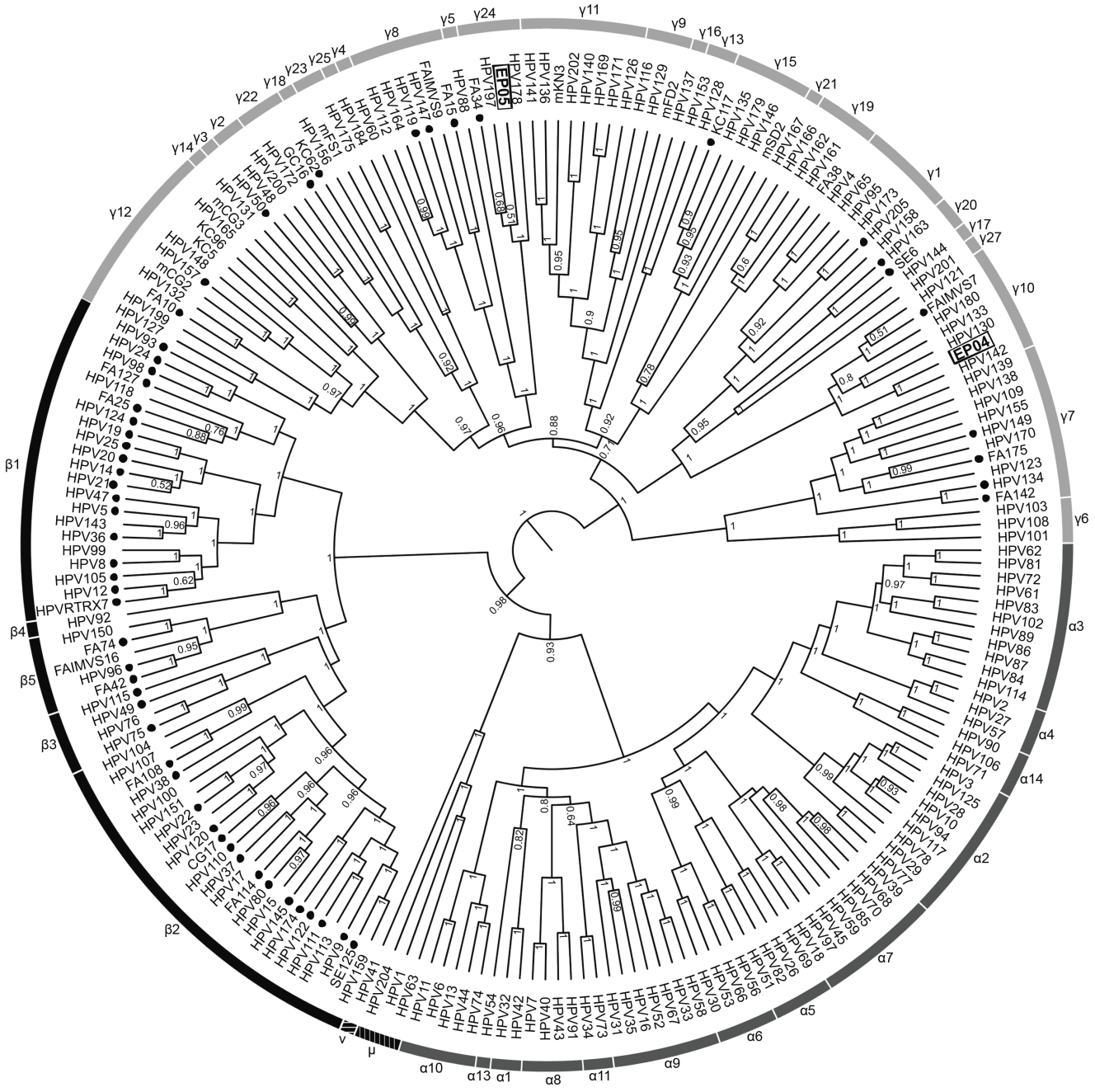

Fig. 2. Phylogeny of HPV types/putative types identified in this study with respect to all officially recognized HPVs. Phylogenetic analysis of partial L1 gene sequences (~370 bp for CUT and ~235 bp for FAP primer pairs) of HPV types/putative types identified in this study and 187 reference HPVs from α-, β-, γ-, μ- and ν-PV genera. Bayesian posterior probability values (BPP) >0.50 are shown. HPV types/putative types identified with FAP (solid dot) and CUT (empty dot) primers are shown. Novel putative HPV types identified in this work are indicated with empty squares.

putative HPV types identified in this study, EP04 and EP05. EP04 belongs to species $\gamma-10$, showing the highest nucleotide identity with HPV142 (83.8 %), and EP05 grouped into species $\gamma-24$, showing the highest nucleotide identity with HPV197 (74.1 %).

HPV prevalence during three climatic seasons

Subsequently, we evaluated the prevalence of HPV infection in each climatic season using a combination of both HPV testing strategies. Table 1 summarizes the main outcomes and detailed data is shown in Table 2.

As shown in Table 1, season-specific prevalences of HPV infection were determined to 54, 45 and 47 % in spring, summer and winter, respectively. Despite the fact that the observed differences were not statistically significant (P=0.43), higher trends of HPV infection and a greater number of HPV types/putative types and species were found in spring as compared to to summer and winter. Moreover, prevalences of mixed HPV infections were significantly higher in spring with respect to summer and winter (P=0.02).

Overall, HPV types belonging to species $\beta-1$ were the most prevalent, as both HPV5 and HPV19 were most frequently found in spring samples (5/48), HPV19 in summer samples (4/31), and HPV5 in winter samples (5/34). A similar proportion of putative HPV types was identified in each period, although the only two novel ones, EP05 and EP04, were found in summer and winter, respectively.

HPV persistence over three climatic seasons

Successively, we analysed the persistence of HPV infection among the 78 included healthy individuals. Overall, 62 individuals (80 %) were infected with HPV in at least one of the climatic seasons and 16 (20 %) were HPV-negative throughout the studied period (Table 2).

In order to analyse the type of infection established by each HPV type/putative type, the following criteria were introduced: (i) a *persistent infection* was defined as the identification of the same HPV type/putative type in at least two climatic seasons as described by others [23]; (ii) a *transient infection* was specified as the identification of an HPV type/putative type in only one climatic season. However, HPV types detected exclusively in winter were not categorized as transient infections due to the lack of follow-up, and were considered as (iii) *indeterminate* infections.

According to these criteria, we identified 24 persistent infections, 84 transient infections and 31 indeterminate infections (Table 2). Among the 62 HPV-infected volunteers, 23 (37 %) had persistent infections, and five of them were infected with the same HPV type/putative type in all climatic seasons. Most persistently infected individuals (22/23; 96 %) had single HPV infections, although we also identified a persistent co-infection with FAIMVS9 and HPV5 (Table 2; volunteer 64).

Table 1. HPV prevalence in healthy sun-exposed skin during three climatic seasons

	Spring	Summer	Winter
HPV prevalence *n* (%)	42 (54)	35 (45)	37 (47)
Mixed infections *n* (%)	18 (23)	6 (8)	11 (14)
Number of HPV types/ putative types	48	31	34
Most prevalent HPV species	$\beta-1$	$\beta-1$	$\beta-1$
Most prevalent HPV types	HPV5, HPV19	HPV19	HPV5
Number of HPV species	17	10	10
Putative HPV types	11	9	8
Novel putative HPV types	None	EP05	EP04

Subsequently, we evaluated whether viruses clustering to α-, β- and γ-PV genera had different capacities to establish persistent infections of the host. With that purpose, we analysed each infection separately. A total of 108 infections were eligible for this analysis (84 transient and 24 persistent infections; Table 2), while indeterminate infections were excluded. Overall, infections with β-PV types/putative types (77/108) were most frequent, followed by γ-PV types/putative types (21/108) and α-PV types (10/108). Seventy-three percent (56/77) of β-PV infections were transient and 27 % (21/77) of β-PVs persisted over time. On the contrary, γ- and α-PV infections seemed to be mainly transient, with approximately 10 % persisting over time (2/21 and 1/10, respectively). However, when comparing proportions of persistent infections caused by β-PVs versus γ- and α-PVs, no statistically significant differences were observed (P=0.15).

Among persistent infections with β-PV types/putative types, those grouped into species $\beta-1$ were the most frequent (12/21) and included 10 different types/putative types (Table 2; HPV5, HPV19, HPV20, HPV21, HPV24, HPV25, HPV36, HPV93, HPV124 and HPVRTRX7). Members of the species $\beta-2$ were the second most common β-PV types involved in persistent infections (6/21), implicating five different types/putative types (HPV37, HPV111, HPV174, FA108 and FA114). HPV types grouped into species $\beta-3$ (HPV75) and $\beta-5$ (HPV96) were identified in 2/21 and 1/21 persistent infections, respectively. Concerning single types, HPV5 was the most frequent virus found in persistent infections (3/21), followed by HPV37 and HPV75 (2/21) (Table 2).

Analysis of HPV infection in relation to demographic and behavioural variables

We analysed demographic (age and gender) and behavioural (estimated time of sun exposure, use of sunscreen and SPF degree) variables in relation to the infection status and persistence of HPV infection. First a univariate analysis comparing the infected (62/78) and uninfected (16/78) volunteers was performed. HPV infection status was not associated with gender, estimated time of sun exposure or the use of sunscreen (P=0.16, P=0.32 and P=0.36, respectively). However, HPV-infected volunteers were significantly older than the HPV-negative individuals (Table 3; median age 39 versus 33 years old, respectively; P=0.02). Multiple logistic regression analysis confirmed the results obtained with univariate analyses, with increasing age being the only variable associated with higher frequency of HPV infection (P=0.01; data not shown).

Subsequently, we investigated whether the mentioned variables were related to the persistence of HPV infection. With that purpose, we used the following criteria to categorize the type of HPV infection in each volunteer: (i) no infection (HPV-negative throughout the study period); (ii) persistent infection (HPV-infected individuals with persistent types as defined above); and (iii) transient/indeterminate infection

Table 2. Overview of HPV types/putative types detected in a total of 234 healthy sun-exposed skin swabs collected from 78 immunocompetent individuals at three climatic seasons

Volunteer Code	Age	Gender	Time of sun exposure (h/week)*	SPF	Spring			Summer			Winter			Infection Status	Types of infection†		
					α	β	γ	α	β	γ	α	β	γ		Persistent (*n*)	Transient (*n*)	Indeterminate (*n*)
1	26	M	2	No					**96**			**96**		P	1		
2	28	F	1	30					**124**			**124**		P	1		
3	29	F	2	30								20	FAIMVS7	P			2
4	39	F	6	30		*FA127*	*FA142*					111		P		2	1
5	30	F	3.5	20										N			
6	34	F	4.5	30		**5**		*2*				**5**, 8		P	1	1	1
7	26	F	4	30								5		P			1
8	35	M	2	50										N			
9‡	33	F	14	15	*2*	**20**			**20**, *36*			**20**		P	1	2	
10	31	M	8	No										N			
11	51	F	5	20					**111**			21, 93, **111**		P	1		2
12	44	F	10	30		*120*							149	P		1	1
13	44	F	5	30		*36*	*205*					21		P		2	1
14	54	F	14	30		*24*						19		P		1	1
15	40	F	7	30								5		P			1
16	52	F	4	No		**75**, *111*						5, **75**		P	1	1	1
17	28	F	3	30										N			
18	26	M	5	15										N			
19	27	M	4	10		*21*								P		1	
20	53	M	5	No	*89, 40*	*22*								P		3	
21	55	M	3	20		*8*, **75**	*FA10*		**75**, *FA74*					P	1	3	
22	60	M	0	No					*49*					P		1	
23‡	33	F	4	30	*78*	**21**	*FAIMVS9*		**21**	*167*		**21**		P	1	3	
24	32	F	15	20		5								P		1	
25	53	F	8	15	*2*		*157, KC62, FA10, KC96, FA38*						SE6	P		6	1
26‡	36	M	11	30		**19**			**19**			**19**		P	1		
27	26	F	4	15		*19*			*FA108*					P		2	
28	23	F	4	No										N			
29	35	M	5	40										N			
30	39	M	12.5	30		*47*						120		P		1	1
31	25	F	8	No	*2*				*19*					P		2	
32	38	F	6	30										N			
33	30	F	4	30										N			
34	28	F	0	No										N			

Table 2. cont.

Volunteer Code	Age	Gender	Time of sun exposure (h/week)*	SPF	Spring			Summer			Winter			Infection Status	Types of infection†		
					α	β	γ	α	β	γ	α	β	γ		Persistent (*n*)	Transient (*n*)	Indeterminate (*n*)
35	50	F	5	30		*21, 37*	*153*							P		3	
36	36	M	6	30	*2*	*98, 107*			*8*					P		4	
37	25	F	0	30		*9*								P		1	
38	52	F	4	20		*20*			**36**			**36**, 124		P	1	1	1
39	47	F	7	15					*19, 25*	*KC117*		38b		P		3	1
40	46	F	15	15							2			P			1
41	35	M	25	20		**174**			**174**				EP04	P	1		1
42	29	M	25	20		*19*								P		1	
43	48	M	0	40		*105*			*120*					P		2	
44‡	35	M	4	20		**5**			**5**			**5**		P	1		
45	27	M	1	No										N			
46	52	F	1	20					*111,* **FA108**	*EP05*		**FA108**		P	1	2	
47	51	F	15	30		*14D, 17, FA108, 120*			**25**			**25**		P	1	4	
48	33	F	3	20										N			
49	28	F	1.5	No		*12, 20, 80*								P		3	
50	37	M	6	15										N			
51	27	M	5.5	40		*110, 115*								P		2	
52	45	F	2	No	**28**			**28**						P	1		
53	52	F	10	No										N			
54	63	F	2	No		*49*	*FAIMVS7*		*FA42*	*GC16*		107, 145		P		4	2
55	41	F	3	20										N			
56	34	M	10	No		*5*								P		1	
57	56	F	4	30		*GC17*	*163*		**FA114**			**FA114**		P	1	2	
58	52	F	0	40			*205*							P		1	
59	41	M	0	No										N			
60‡	53	F	0	No		**RTRX7**	*50*		**RTRX7**			**RTRX7**		P	1	1	
61	29	F	0	20		*107*								P		1	
62	58	F	1	15		*SE125*			**37**			21, **37**		P	1	1	1
63	58	F	0	No		*19*								P		1	
64	45	F	0	No	3	**5**, *20*	**FAIMVS9**		**5**, *21*				**FAIMVS9**	P	2	3	
65	58	F	3	15		*19*								P		1	
66	29	F	0	20			**50**		*20*				**50**	P	1	1	

Table 2. cont.

Volunteer Code	Age	Gender	Time of sun exposure (h/week)*	SPF	Spring			Summer			Winter			Infection Status	Types of infection†		
					α	β	γ	α	β	γ	α	β	γ		Persistent (*n*)	Transient (*n*)	Indeterminate (*n*)
67	32	F	1.5	30		**37**						**37**, 119		P	1		1
68	29	F	7.5	35		*38b, 98*								P		2	
69	27	F	5	8					*24*			107		P		1	1
70	29	F	2	30						*FA34*			50	P		1	1
71	30	M	3	30					*FA25*					P		1	
72	39	F	0	No					*19*					P		1	
73	55	F	0	No					*20*					P		1	
74	54	F	6	25					*80*					P		1	
75	39	F	0	50					**24**			12, **24**		P	1		1
76	31	M	5	20								24	147, FA175	P			3
77	29	F	9	40								111	134, FA15	P			3
78	52	F	7	20					**93**			**93**		P	1		
Total						**42**			**35**			**37**			**24**	**84**	**31**

HPV types/putative types identified in each climatic season are presented by genera. Numbers correspond to types officially designated at the International HPV Reference Center (www.hpvcenter.se/), while codes relate to putative HPV types, types identified by metagenomic sequencing and other types deposited at http://pave.niaid.nih.gov/ [4].

HPV types/putative types involved in persistent infections are depicted in bold and those participating in transient infections are shown in italics.

*Estimated mean time of sun exposure in spring and summer declared by each volunteer in the questionnaire.

†According to the criteria defined in the text.

‡Volunteers with persistent infections with the same type in all studied climatic seasons.

F, female; M, male; N, negative; P, positive; SPF, sun protection factor.

Table 3. Associations of demographic and behavioural variables with the acquisition and persistence of HPV infection in sun-exposed healthy skin of immunocompetent individuals

Variable	Infection status		P	Type of infection*			P
	HPV-negative (*n*=16)	HPV-positive (*n*=62)		No infection (*n*=16)	Transient/indeterminate (*n*=39)	Persistent (*n*=23)	
Gender (%)							
Male	30	70	0.16	30	48	22	0.32
Female	16	84		16	51	33	
Median age (years)	33	39	**0.02**	33‡	39	45‡	**0.05**
Median time of sun exposure (h/week)	4	4	0.32	4	5	4	0.41
Use of sunscreen (%)†							
Low	18	82	0.36	18	64	18	0.34
Medium	15	85		15	46	39	
High	25	75		25	63	12	
Not used	30	70		30	45	25	

*Individuals with persistent and transient/indeterminate infections were considered as 'persistent'.

†Use of sunscreen was classified as: low (8–15 SPF), medium (20–30 SPF), high (35–50 SPF) and not used.

‡Significant differences. *P* values≤0.05 are depicted in bold.

(HPV-infected individuals without persistent infections). No significant associations were observed between gender, estimated time of sun exposure, use of sunscreen or persistence of HPV infection (Table 3). Yet, HPV-infected individuals with persistent infections were significantly older than HPV-negative individuals (*P*=0.05), although they were not significantly different in age from HPV-infected individuals with transient/indeterminate infections (Table 3). Multiple logistic regression analysis confirmed that age was associated with the type of HPV infection (no infection, indeterminate/transient, persistent) in healthy skin (*P*=0.02; data not shown). Namely, uninfected volunteers were younger and the potential to obtain transient/indeterminate and persistent infections increased with increasing age.

Characterization of HPV209, a novel HPV type clustering to species β−2

The complete genome sequence of the putative HPV type FA108 (Accession no. AY20469), involved in transient and persistent infections in the present study, was characterized from a swab sample collected in spring (Table 2 volunteer 47). Using the 'hanging droplet' long-range PCR technique [27], two overlapping genomic halves of the novel virus were amplified, cloned and sequenced, and the complete viral genome was officially designated as HPV209 (see Methods).

The full genome of the novel virus was determined to be 7399 bp in length with a GC content of 40.4 %. The *in silico* analysis of the HPV209 genome showed the typical genomic organization of cutaneous HPVs (Table 4), potentially encoding five early (E6, E7, E1, E2 and E4) and two late genes (L2 and L1), as well as missing an E5 ORF, which is a characteristic feature of human β-, γ-, μ- and ν-PVs [27].

Within the upstream regulatory region (URR) of HPV209, a typical TATA box, three putative binding sites for the E2 protein, a binding site for the E1 protein [28] and a putative polyadenylation site for late gene transcripts were identified (Table 4). 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).

Typical domains were additionally identified in the putative viral proteins of HPV209. The E6 ORF contained two characteristic zinc-binding domains separated by 36 aa [29] and a PDZ-binding motif (RTIL) [30]. The E7 ORF of HPV209 contained a pRB binding motif (LHCYE) [31] and a single zinc-binding domain. Analysis of the putative E1 gene product, the largest protein encoded by HPV209 (Table 4), showed the typical ATP-binding site of the ATP-dependent helicase (GPPDSGKS) [32], and several cdk-phosphorylation and cyclin-binding sites. At the N-terminal region of the E1 protein, a highly conserved bipartite-like nuclear localization signal (NLS) and a leucine-rich Crm1-dependent nuclear export signal (NES) were identified, which together enable shuttling of the E1 protein between the cell nucleus and cytoplasm in most HPVs (Table 4) [33–35]. The putative HPV209's E4 ORF is typically positioned within the E2 ORF (2480–3853 nt) and has its own start codon and a characteristic conserved leucine motif (LLLLL) important for keratin association. Moreover, a typically high proline content (13.4 %) with an important role in cell cycle arrest was found in the E4 protein [36].

At the N-terminal region of the L2 protein, a highly conserved furin cleavage motif (RTKR) as well as a transmembrane-like domain (GISTGKGTGGTTGYVPLGEGLGVRVG) [37] were identified (Table 4). In addition, a single canonical polyadenylation site, necessary for regulation of early viral transcripts [38], was also found at the L2 protein's N-terminus (Table 4).

Phylogenetic analyses and pairwise comparisons of L1 ORFs confirmed HPV209 as a novel member of the β-PV genus (Fig. 3), with nucleotide identities with viruses

Table 4. Main genomic features, putative proteins and nucleotide identities of HPV209 with HPV types representing β-PV species

Putative ORFs	length (nt)	Nucleotide sequence (pre-stop codon) (nt)	Protein size (aa)	HPV motifs and domains (consensus sequence)	Genomic position	Nucleotide identity (%)				
						HPV107 ($\beta-2$)	HPV49 ($\beta-3$)	HPV92 ($\beta-4$)	HPV5 ($\beta-1$)	HPV96 ($\beta-5$)
URR	400	7000–7399	–	Polyadenylation site (AATAAA)	nt 7018–7023	77.2	49.1	48.0	40.9	45.6
				TATA box (TATAAA)	nt 7366–7371					
				E1 binding site (ATTGTGGTTTACAACTA TCAT)	nt 7307–7327					
				E2 binding site (ACC(N)$_6$GGT)	nt 7102–7113					
					nt 7245–7256					
					nt 7274–7285					
E6	423	1–423	140	Zinc binding domain (CXXC(X)$_{29}$CXXC)	aa 27–63	91.1	58.6	57.1	52.9	55.3
					aa 100–136					
				PDZ binding domain (X(T/S)X(L/V))	aa 7–10					
E7	308	420–728	102	Zinc binding domain (CXXC(X)$_{29}$CXXC)	aa 57–93	95.1	68.9	65.8	63.6	67.1
				pRB binding site (LXCXE)	aa 25–29					
E1	1823	715–2538	607	Bipartite-like NLS (KRK(X)$_{25}$KKRL)	aa 79–81…107–110	92.3	68.9	69.3	67.7	69.3
				NES (L(X)$_{2-3}$L(X)$_2$(L,I,V)X(L,I))	aa 91–100					
				ATP binding site (GXXXXGK(T/S))	aa 434–441					
				cdk phosphorylation site ((S/T)P)	aa 84–85					
					aa 92–93					
					aa 101–102					
				Cyclin binding motif (RXL)	aa 60–62					
					aa 248–250					
					aa 534–536					
E2	1373	2480–3853	457	Leucine zipper domain (L(X)$_6$L(X)$_6$L(X)$_6$L)	Absent	94.2	64.9	66.5	61.0	65.2
E4	608	3000–3608	202	Leucine motif (LLXLL)	aa 22–26	93.8	66.7	62.5	58.3	60.4
L2	1559	3905–5464	519	Polyadenylation site (AATAAA)	nt 3995–4000	88.1	67.3	67.4	65.7	69.9
				Furin cleavage motif (RX(K/R)R)	aa 3–6					
				Transmembrane-like domain (G(X)$_{26}$G)	aa 56–81					
L1	1523	5476–6999	507			88.1	73.1	70.8	69.9	69.2

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

representing current β-PV species ranging from 69.2 to 88.1 % (Table 4). This novel HPV type represents the 20th member of the species $\beta-2$, showing the highest identity to HPV107 along the whole genome (Table 4). Moreover, HPV104, HPV107 and HPV209 are grouped in a separate monophyletic clade with respect to the other members of the $\beta-2$ species (Fig. 3). The comparative analysis of genomic motifs and domains between the three phylogenetically- related viruses indicated that they have similar characteristics (data not shown).

DISCUSSION

Contrary to mucosal HPV types, knowledge about the natural history of cutaneous HPV infections is limited. Owing to the paucity of evidence, the International Agency for Research on Cancer has not yet been able to classify cutaneous HPVs according to their oncogenicity [39]. Nevertheless, it has been proposed that some β-PVs act as co-factors together with UV radiation early in the cutaneous carcinogenic process [18].

Most of the UV-B radiation (280–320 nm) passing through the atmosphere is filtered by stratospheric ozone. As stratospheric ozone decreases, higher levels of UV-B reach ground level [26]. To our knowledge, this is the first longitudinal study reporting the characteristics of HPV infection over three climatic seasons in sun-exposed skin of healthy individuals living in a geographical area in which solar UV radiation is influenced by the ozone content of the atmosphere. Interestingly, in comparison to other climatic seasons, frequencies of overall HPV infection (P=0.43), mixed infections (P=0.02), the number of HPV types/putative types and species were higher in spring (Table 1), coinciding with the ozone hole passing over Argentina (Fig. S1, available in the online Supplementary Material) [26]. These trends may indicate an influence of the composition of solar radiation on HPV infection.

A few reports have explored the prevalence of HPV infection in healthy sun-exposed skin in different geographical regions [24, 40–42], with values ranging from 42 to 70 %. Most of them used single-testing strategies [24, 40, 41] or assays directed to particular PV genera [42]. In our study, we used a combination of CUT and FAP primer systems, enabling amplification of most cutaneous HPV types [16]. With these tools, we could identify a total of 71 HPV types/putative types from three out of the five genera in which cutaneous HPVs are distributed (Fig. 2). In addition, in all three analysed climatic seasons, the overall HPV prevalences (54 % in spring, 45 % in summer, 47 % in winter; Table 1) were within the reported range [24, 40–42]. However, the proportion of individuals with persistent infections detected in our population (37 %, 23/62) was lower than previously reported [23, 24], albeit fewer individuals were included in those studies. In addition, the use of broad-spectrum PCR primers like FAP and CUT may lead to a competition among different HPV types where the minor HPV type could be out-competed and remain unidentified [43]. This phenomenon is a common problem in broad-spectrum PCRs and could underestimate the number of HPV types within the same sample [23]. Overall, 35 samples with mixed infections were identified (Table 1); however, only three clones per sample were analysed using DNA sequencing. These technical limitations may be reflected in 5/23 individuals with persistent infections (Table 2; participants 6, 16, 64, 66, 67) where the same HPV type/putative type was detected in spring and winter but not in summer. Also, the low frequency of persistent co-infections found in this group of individuals (Table 2; participant 64) may be due to the aforementioned limitations. The application of next generation sequencing techniques could enable a more in-depth analysis of all HPV types involved in mixed infections.

It has been demonstrated that the persistence of mucosal high-risk HPVs is a key factor in the development of cervical cancer [44], but little is known on the persistent infections of cutaneous HPVs. In this study, we have determined that 80 % (62/78) of the volunteers were positive for HPV DNA in at least one of the seasons and 37 % of them had a persistent HPV infection (22 volunteers with single infections and one with a co-infection). When comparing the age of individuals according to the type of infection (Table 3), the persistently infected subjects were significantly older than the HPV-negative ones. Multivariate analysis confirmed that age was associated with both the infection status and the type of HPV infection. Our findings are in agreement with previous reports [45–47], which suggested that increasing age may be an HPV infection-associated factor, particularly for infections with HPV types of the β-PV genus. In fact, it has been proposed that an effect named immunosenescence could be implicated in this association [48]. As a result, the deterioration of the immune system with increasing age might therefore be the underlying mechanism explaining both the increasing prevalence and the persistence of cutaneous HPV infection with age [45, 47]. On the other hand, we were not able to establish associations between the other studied variables (gender, estimated time of sun exposure and use of sunscreen), neither with the HPV infection status nor with the persistence of HPV infection (Table 3). Other studies have shown that gender was not associated with cutaneous HPV infection or its persistence [10, 24, 45]. Also, in agreement with other studies, the estimated time of sun exposure [45] and use of sunscreen [41] were not found to be associated with HPV infection. However, Chen *et al.* have shown that there was a direct relationship between increased time spent outdoors and detection of HPV DNA in a population of healthy individuals with and without frequent sun exposure in Australia, a strong UV-radiation region comparable to Argentina [41]. Instead, the individuals included in our study population mostly had indoor jobs and therefore we cannot exclude the possibility that the time of sun exposure plays a role in cutaneous HPV prevalence or persistence in normal skin.

Overall, β-PV types/putative types were not only the most frequently identified types in each climatic season, but also caused the highest proportion of persistent infections (Tables 1 and 2). Our data did not reach statistical

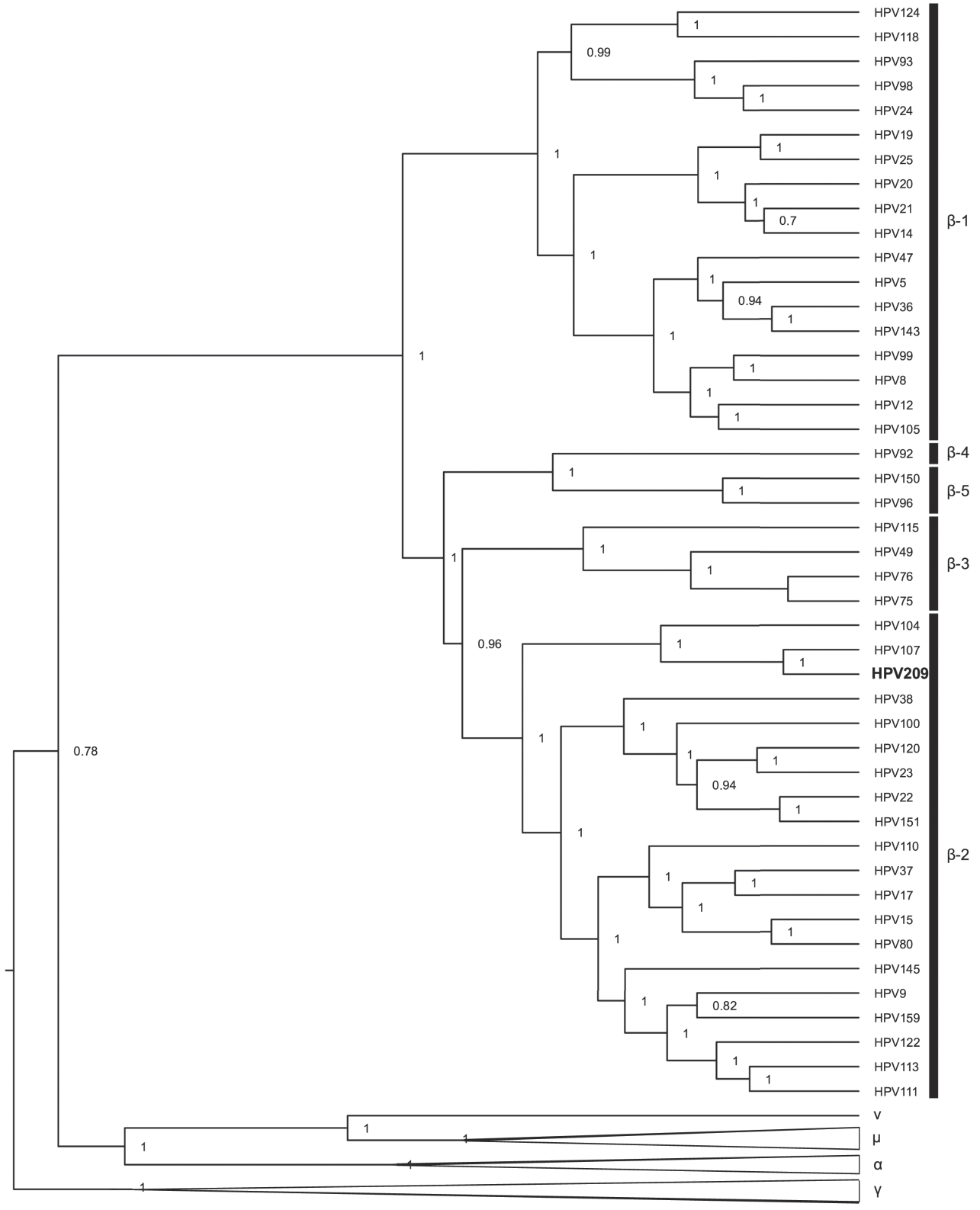

Fig. 3. Phylogeny of HPV209 with respect to reference HPVs. Phylogenetic analysis of L1 ORF sequences of the novel HPV type characterized in this study and reference HPVs from α-, β-, γ-, μ-, and ν-PV genera. Bayesian posterior probability values (BPP) >0.50 are shown. Novel HPV type HPV209 is shown in bold. α-, γ-, μ- and ν-PV genera branches are collapsed.

significance with respect to the dissimilar capabilities of members of different PV genera to establish persistent infections (P=0.15). However, we found that infections involving β-PV types were more prone to persist than those associated with α- or γ-PV types/putative types (27 %10 %). This tendency is unlikely to be attributable to eventual differences in sensitivities to specific genera in the PCR detection systems, since γ- and α-PV types/putative types were also detected (Table 2). Furthermore, it has been shown that CUT primers are more specific in detecting α- and γ-PV types [16, 49], whilst FAP primers perform better in the detection of β-PV types [16]. In fact, the general concordance of both systems was low (57.7 %) [κ=0.0907; IC95 %: 0.0086-0.1729], with only six types/putative types detected by FAP and CUT primers simultaneously. On the other hand, γ-PV and α-PV types were mainly involved in transient infections (Table 2). Interestingly, Deng *et al.* have recently proposed that frequencies of γ- and α-PV types gradually decrease with increasing age, in contrast to the observed β-PV types [10]. This finding reinforces our observations regarding the tendency of β-PVs to persist. However, a larger sample size would be needed to elucidate whether members of γ- and α-PV genera may establish persistent infections of the skin.

HPV5 ($\beta-1$) was the most frequently identified type overall as well as the most persistent HPV type (Tables 1 and 2). Despite the fact that epidemiological data about cutaneous HPVs are controversial and heterogeneous, many studies have proposed that HPV types from species $\beta-1$, including HPV5, are the most prevalent and persistent HPV types identified in both healthy skin and in HPV-related cutaneous lesions [24, 50–52]. In addition, Antonsson *et al.* analysed a set of skin swab samples from healthy individuals from three continents and determined that HPV5 was the most prevalent virus and the only HPV type found in all studied countries [40]. An important point worth noting is that members of the species $\beta-1$, possibly activated by UV radiation [20], are considered a risk factor for the development of NMSC in solid organ transplant recipients and in epidermodysplasia verruciformis patients [19]. Therefore, a persistent infection with these β-PV types should represent a higher risk for the development of cutaneous lesions [21]. Nevertheless, in this study, the relation between the virus and the host was not investigated, although it seemed to be commensal, in agreement with other reports [23, 24].

A large genetic diversity within cutaneous HPVs has been reported, with most putative γ-PV types segregating outside the defined species [8]. In this study, we identified two novel putative γ-PV types, EP04 and EP05, and we fully characterized the genome of HPV209, a novel type of $\beta-2$ species. Future studies are needed to determine the clinical significance of members of the $\beta-2$ species infections, taking into account the high prevalence of these types, including HPV209 (formerly putative HPV type FA108), in cutaneous squamous cell carcinoma and actinic keratosis [53–55]. Moreover, the presence of a pRB binding motif in the E7 ORF of HPV209 might indicate transforming properties of this novel β-PV type. On the other hand, the novel HPV isolate EP05 seems to be a novel potential member of the recently recognized species $\gamma-24$ (Fig. 1), in which the viral prototype HPV197 has been shown as the most frequently detected HPV type in skin tumour specimens [56]. A recent proteomic analysis has revealed that its E6 and E7 proteins interact with important cellular targets of mucosal and/or cutaneous HPV oncoproteins [57], adding new questions about the role of cutaneous types in the oncogenic process.

In conclusion, this longitudinal study provides valuable data about the characteristics of HPV infection in healthy individuals living in a region in which solar UV radiation is influenced by the ozone content in the atmosphere. We determined that older age is a risk factor for both HPV positivity and persistence, confirming the need sun protection campaigns to prevent viral infections and cumulative UV damage of the skin. The characterization of HPV209, a novel member of β-PV species 2, expands the current knowledge of PV diversity. Altogether, these findings will be useful in the elucidation of the possible role of cutaneous HPVs in human skin pathologies in high UV-exposure regions.

METHODS

Patients and data collection

To address the HPV prevalence and persistence in sun-exposed healthy skin of immunocompetent individuals, a longitudinal study was conducted between October 2013 and August 2014. Recruitment targeting university students, faculty, staff and members of the general population started in August 2013 by word-of-mouth communication and through posters located inon a school campus.

Inclusion criteria were: (i) age $\geq$18 years and (ii) residence in Rosario city or in the Greater Rosario area for at least the duration of the study. Exclusion criteria were medical history of skin pathology, HIV infection and immunosuppressive therapy. Forehead swab samples were collected from every volunteer in three different climatic seasons: spring (October 2013), summer (March 2014) and winter (August 2014).

All subjects provided informed consent and were interviewed to obtain basic demographic information (age and gender), skin type (I–VI) according to the Fitzpatrick classification [58], estimated time of sun exposure in spring and summer (hours per week), use of sunscreen and degree of used sun protection factor (SPF).

Sample collection and processing

Superficial cells on the forehead of each volunteer were sampled with a sterile cotton-tipped swab pre-wetted in saline solution (0.9 % NaCl). Swabs were drawn back and forth (10 times) over the skin surface within an area of 5×5 cm, and were then suspended in 1 ml saline solution. Each swab was agitated, discarded and the samples were further processed, as described previously [16]. Briefly, the cells were centrifuged at 7000 ***g*** for 2 min and pellets were resuspended in 100 µl TE

buffer containing 100 µg proteinase K and incubated overnight at 55 °C. After protnase K inactivation at 95 °C for 10 min, cell lysates were stored at −20 °C until analyses.

Identification of HPV infection

The presence of HPV infection was determined using previously described generic primer systems, FAP [59, 60] and CUT [16, 49], using a single tube 'hanging droplet' nested PCR technique [16]. All PCR reactions were performed as described previously [16] using the thermocycler Mastercycler Personal (Eppendorf, Germany). PCR amplicons were purified with spin columns (NucleoSpin Extract II kit; Macherey-Nagel, Germany) and DNA sequencing was performed at the sequencing facility (Macrogen, USA). If the electropherogram suggested the presence of a mixed HPV infection, the pGEM-T Easy Cloning kit (Promega, USA) was used to clone the questionable PCR amplicons. At least three recombinant clones were sequenced from each sample. The obtained sequences were compared to HPV nucleotide sequences available in the GenBank database (www.ncbi.nlm.nih.gov/) using the BLAST algorithm.

Identification of the full-length genome sequence of HPV209

HPV209, previously known as isolate FA108, was identified in a swab sample collected from a 51 year old female. 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 [27]. One half of the viral genome was amplified with the E1Beta-R (5′-tcttgyagwrcaytgaaacg-3′)/FAP6085-F [59] generic primer pair and the other overlapping amplicon was obtained with specific primers V77-F (5′-cagcccctatcagaaactaaactt-3′) and V77-R (5′-ctacgcaggcccatactaatct-3′). Both specific primers were designed based on 3′ and 5′ regions of the genomic half obtained with generic primers using the FastPCR program [61]. Reaction mixtures and cycling conditions were the same as described previously [62]. The annealing temperature of the V77-1_F/V77-1_R primer pair was 50 °C in both rounds of amplification.

Bands of approximately 4 kbp were excised from the gel and purified using a NucleoSpin Extract II kit (Macherey-Nagel, Germany). The amplicons generated with generic and specific primers were cloned using the pGEM-T Easy Cloning kit (Promega, USA) and the TOPO XL PCR Cloning Kit (Invitrogen, USA), respectively. DNA sequencing was performed at the sequencing facility (University of Maine DNA Sequencing Facility, USA). The obtained nucleotide sequences were compared to HPV sequences available in the GenBank database (www.ncbi.nlm.nih.gov/) using the BLAST server. In August 2015, the reference clones and corresponding nucleotide sequences of a putative novel HPV type were submitted to the Human Papillomavirus Reference Centre at the Karolinska Institutet in Sweden, where its nucleotide sequence was confirmed and the official number HPV209 was assigned in February 2016 (www.hpvcenter.se/html/refclones.html).

Phylogenetic analysis

Multiple sequence and pairwise alignments at the nucleotide level were constructed using the CLUSTALW algorithm of the MEGA6 software package [63]. Sequences from representative HPV types are available at www.hpvcenter.se/ and http://pave.niaid.nih.gov/ [4]. For phylogenetic analysis of HPV209, L1 gene sequences from 187 representative HPV types were used. For the phylogenetic analysis of novel (EP04 and EP05) and previously identified putative HPV types, the analysis additionally included the corresponding FAP and CUT amplicons. Novel putative HPV types were defined according to the current HPV classification criteria based on nucleotide identities in the L1 gene sequences [1].

The phylogenetic relationships were inferred by Bayesian analysis using BEAST version 1.7.5 [64]. In order to do this, Markov-chain Monte Carlo (MCMC) simulations were performed during 2×10^7 generations, sampling one state every 1000 generations, with a burnin of 10 %. Statistical convergence of the MCMC simulations was assessed visually by the traceplot and by calculating the effective sample size using Tracer version 1.4 (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.

Statistical analysis

The concordance between the CUT and FAP primer systems in detecting HPV infections in all samples included in the study was determined by thekappa coefficient (κ).

The comparison of the overall prevalence of HPV infection and mixed infections across distinct climatic seasons was performed using Cochran's Q test.

Statistical analysis of categorical variables (gender and use of sunscreen) was performed by chi square, meanSscore and exact Ccorrelation tests. The comparison of means (age and time of sun exposure, hours/week) was made through Kruskal–Wallis and Mann–Whitney's tests. *P* values below 0.05 were regarded as statistically significant.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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 (reference number 6060/134). All subjects provided informed consent to participate in the study.

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