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

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This study developed a hanging-droplet long PCR, a generic and highly sensitive strategy to facilitate the identification of new human papillomavirus (HPV) genomes. This novel procedure used for the first time the hanging-droplet PCR technique for the amplification of long DNA fragments with generic primers targeting the L1 and E1 regions. It was first applied to the amplification of types belonging to the highly divergent genus *Gammapapillovirus* (γ-PV). The hanging-droplet long PCR was 100-fold more sensitive than a simple long PCR procedure, detecting as few as ten copies of HPV-4. Nineteen skin samples, potentially containing putative HPV types from the γ-PV genus, were also screened. The method identified four γ-PV genomic halves from new and previously described putative types, and made the full characterization of HPV-156 possible. This novel virus meets the criteria for a new species within the γ-PV genus, with nucleotide identities in the L1 ORF ranging from 58.3 to 67.3 % compared with representative types of the current γ-PV species. HPV-156 showed the highest identity to HPV-60 (67.3 %) from species γ-4, and was consistently closely related to it in both late- and early-gene-derived phylogenies. In conclusion, this report provides a versatile and highly sensitive approach that allowed identification of the prototype of a new species within the γ-PV genus. Its application with primers targeting the different genera in which both human and non-human PVs are distributed may facilitate characterization of the missing members of the family *Papillomaviridae*.

INTRODUCTION

Papillomaviruses (PVs) are small, non-enveloped DNA tumour viruses with a circular genome of nearly 8 kb. PVs are principally host specific and have been found in >20 different mammalian as well as bird and reptile species (de Villiers *et al*., 2004; Herbst *et al*., 2009). By convention, a novel PV type shows <90 % nucleotide identity in the L1 gene with respect to any known PV type, whilst PV types belonging to new species within a genus share 60–70 % nucleotide identity with PV types of any other known species (de Villiers *et al*., 2004). Definition of a new PV type requires isolation and characterization of the full-length

genome in order to verify the typical genetic organization of PVs (de Villiers *et al*., 2004).

Presently, >150 human PV (HPV) types have been completely sequenced and have been divided into five genera (α-, β-, γ-, μ- and η-PVs) according to the phylogenetic relationships of their complete L1 gene sequences (de Villiers *et al*., 2004). HPVs are further classified into mucosal/genital and cutaneous types based on sequence analyses and clinical manifestations (Bernard *et al*., 2010). Mucosal/genital HPV types belong to the α-PV genus and have been divided into low-risk and high-risk types as inferred from their association with benign and malignant anogenital lesions, respectively (zur Hausen, 2002). Cutaneous HPV types represent ~75 % of the HPVs described to date, outnumbering those with mucosal/genital tropism (Bernard *et al*., 2010). In addition, skin HPV types are more genetically divergent and are distributed into the

five genera in which HPVs are grouped (Bernard *et al.*, 2010). In contrast to the mucosal/genital HPVs, the involvement of cutaneous HPV types in human carcinogenesis is still unclear. In fact, cutaneous HPVs have been found in benign skin warts and in non-melanoma skin cancer but also on healthy skin (Antonsson *et al.*, 2000). Although the evolutionary basis for the larger genetic diversity of the HPV cutaneous types is unknown, it is speculated that UV light-induced damage may contribute to a higher mutation rate of the HPVs infecting the sun-exposed human skin (Forslund, 2007).

Among the cutaneous HPV types, the γ-PV genus seems to be highly divergent, as inferred from generic primer PCR approaches (Antonsson *et al.*, 2000; Forslund, 2007). More than 30 γ-PV types have been isolated from humans so far (http://pave.niaid.nih.gov), but it is assumed that a much greater number exist in nature. In addition, many novel HPV types/putative types are found segregated outside the current species (Chouhy *et al.*, 2010; Forslund, 2007), indicating that additional species within the genus γ-PV need to be defined. Efforts directed towards identifying novel HPV types are needed in order to have a complete picture of the γ-PV genus to elucidate the evolution and medical implications of these viruses.

For the past 15 years, several PCR-based methods have been used for the identification of HPV DNA in skin lesions (Asgari *et al.*, 2008; Berkhout *et al.*, 2000; Chouhy *et al.*, 2010; Forslund *et al.*, 2007, 2003; Harwood *et al.*, 2000, 2004; Shamanin *et al.*, 1994, 1996), as well as in normal skin (Antonsson *et al.*, 2000; Chen *et al.*, 2008; Chouhy *et al.*, 2010; Hazard *et al.*, 2007). Overall, 200–250 putative HPV types have been identified in the form of PCR amplicons of 200–450 bp (Antonsson *et al.*, 2000; Chouhy *et al.*, 2010; Forslund, 2007, Forslund *et al.*, 1999). However, <25 % of these putative HPV types have been fully cloned and characterized using PCR, rolling-circle techniques or a combination of both methods (Chen *et al.*, 2007; de Villiers & Gunst, 2009; Köhler *et al.*, 2011). The low amplification efficiency of these techniques for long DNA fragments together with the presence of HPV DNA in low copy numbers in skin samples (Vasiljevic *et al.*, 2008; Weissenborn *et al.*, 2005) may have influenced the identification of the remaining putative HPV types. Taking this into account, and in order to provide a generic and highly sensitive method to facilitate the identification of new HPV genomes, we combined the hanging-droplet PCR technique with generic primers targeting the L1 and E1 regions for the amplification of long DNA fragments. This novel method, which we named 'hanging-droplet long PCR', was applied to the amplification of types belonging to the highly divergent γ-PV genus (Forslund, 2007). This PCR strategy allowed the identification of four γ-PV genomic halves from new and previously described putative types, and made possible the full characterization of HPV-156, a novel virus that meets the criteria for a new species within the γ-PV genus.

RESULTS

Hanging-droplet long PCR method

The hanging-droplet long PCR method was designed to facilitate the amplification of long DNA fragments with higher sensitivity than conventional PCR methods used for the identification of novel PV genomes. To achieve this goal, a two-step PCR amplification with the single-tube 'hanging-droplet' PCR strategy (Walsh *et al.*, 2001) was applied, using the same primer pair in both rounds of amplification. A combination of generic primers located within the L1 and E1 gene regions (primer pairs L1-F/E1-R and E1-F/L1-R, Fig. 1a) was used to generate two overlapping amplicons of ~4000 bp corresponding to HPV genomic halves. It should be noted that this strategy can be used with different generic primer pairs. In this work, the widely used FAP primers (Forslund *et al.*, 2003) were combined with those designed in the E1 gene region for the amplification of γ-PVs. After the optimization process, the sensitivity of the new method was compared with an optimized protocol using simple long PCR. A representative experiment is shown in Fig. 1(b). The hanging-droplet long PCR detected down to ten copies of cloned HPV-4, and therefore was 100 times more sensitive
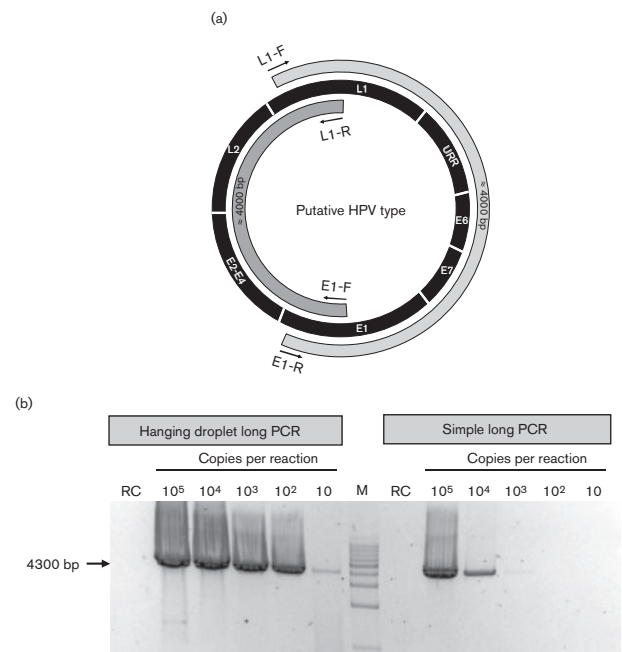

Fig. 1. The hanging-droplet long PCR strategy. (a) General strategy for generating HPV DNA genomic halves. The generic primers used in this work were FAP6085-F (=L1-F), FAP6319-R (=L1-R), E1Gamma-F (=E1-F) and E1Gamma-R (=E1-R). (b) Analytical sensitivity comparison of hanging-droplet long PCR and simple long PCR. PCR products from each PCR strategy were generated with the E1Gamma-R/FAP6085-F primer pair from a tenfold dilution series of cloned HPV-4 in a background of 5 ng human placental DNA (Sigma). RC, Reaction negative control; M, 1 kb ladder (PB-L).

than the simple long PCR procedure using 40 cycles, which was able to amplify 1000 plasmid copies. The same sensitivity was obtained with the simple long PCR procedure when the number of cycles was 65, the same as used in the hanging-droplet long PCR (data not shown), confirming the increase in sensitivity of our novel strategy.

Skin sample screening by hanging-droplet long PCR and phylogenetic analysis

Next, 19 skin samples potentially containing one or more putative HPV types from the γ-PV genus (Chouhy et al., 2010) were screened with the hanging-droplet long PCR using the E1Gamma-F/FAP6319-R and E1Gamma-R/FAP6085-F primer pairs. Amplicons ranging from 3800 to 4400 bp in size were obtained from five of the 19 samples for the first primer set and four of the 19 samples for the second primer set (Table 1). Five of these could be cloned and were identified as HPV genomic halves corresponding to the previously characterized HPV-23 (β-PV species 2), two putative HPV types described previously as 230–370 bp PCR fragments (GC01 and GC10) (Chouhy et al., 2010) and two novel putative HPV types (GC23 and GC24). Phylogenetic analysis based on partial L1 sequences of the GC01, GC10, GC23 and GC24 putative HPV types grouped them into the γ-PV genus (Fig. 2). Pairwise comparisons of types representing

each species allowed us to assign only the GC23 isolate to species 1 (72.4 % nucleotide identity with HPV-4) (Table 2). In contrast, the GC01 and GC24 isolates (74.6 % nucleotide identity between them) qualified as members of a new species according to the current criteria of PV classification (<71 % nucleotide identity with the HPV types representing each species), being closely related to HPV-60 (γ-4) (Fig. 2). The same was true for the GC10 isolate (Table 2). In this case, the closest related types to GC10 were the recently reported HPV-135 and HPV-146 (sequence identities of 67.3 and 63.2 %, respectively) awaiting species designation (Fig. 2).

Identification of HPV-156, a prototype of a novel species within the γ-PV genus

We started characterizing the full-length genome of the GC01 isolate from a sun-exposed healthy skin sample (Table 1, patient 89). The amplicon generated with the FAP6085-F/E1Gamma-R primer pair was unable to be cloned and identified. In view of this, the GC01 subgenomic sequence that was amplified with the E1Gamma-F/FAP6319-R primer pair was used as template to design specific primers to generate the corresponding overlapping fragment (see Methods). Using this procedure, the other genomic half of the novel virus was amplified, cloned and sequenced, and the complete genome of HPV-156 was obtained.

Table 1. Skin samples screened with the hanging-droplet long PCR

Patient ID	Sample type*	Putative HPV types originally detected†	FAP6085-F/E1Gamma-R primers‡	E1 Gamma-F/FAP6319-R primers‡
00	A	GC15	N	N
12	A	FA27, GC04	N	P
17	C	FA150	P	HPV-23
22	A	GC20, FA91	N	GC24
42	C	FA89, FA66, GC11	N	N
42	D	FA89, FA66	N	N
43	A	FA91	N	N
44	A	FA91, GC12.1	N	N
44	C	FA91	N	N
46	A	GC07.2, GC16	N	N
54	A	FAIMVS9	N	N
55	C	GC04	N	N
59	A	GC16	N	N
79	A	FA8	N	N
82	A	GC01	N	N
89	A	GC01, GC12.1, GC13	P	GC01
93	B	GC19, FA66	P	N
95	A	GC08	GC23	GC10
98	C	FA97	N	N

*All samples were collected from superficial cells in the corresponding areas with sterile cotton-tipped swabs pre-wetted in saline solution (0.9 % NaCl) (Chouhy et al., 2010). A, Healthy skin from a sun-exposed area (forehead); B, healthy skin from a non-sun-exposed area (upper inner arm); C, perilesion area; D, lesion area.
†Screening was carried out using CUT and FAP primer systems, as described previously (Chouhy et al., 2010).
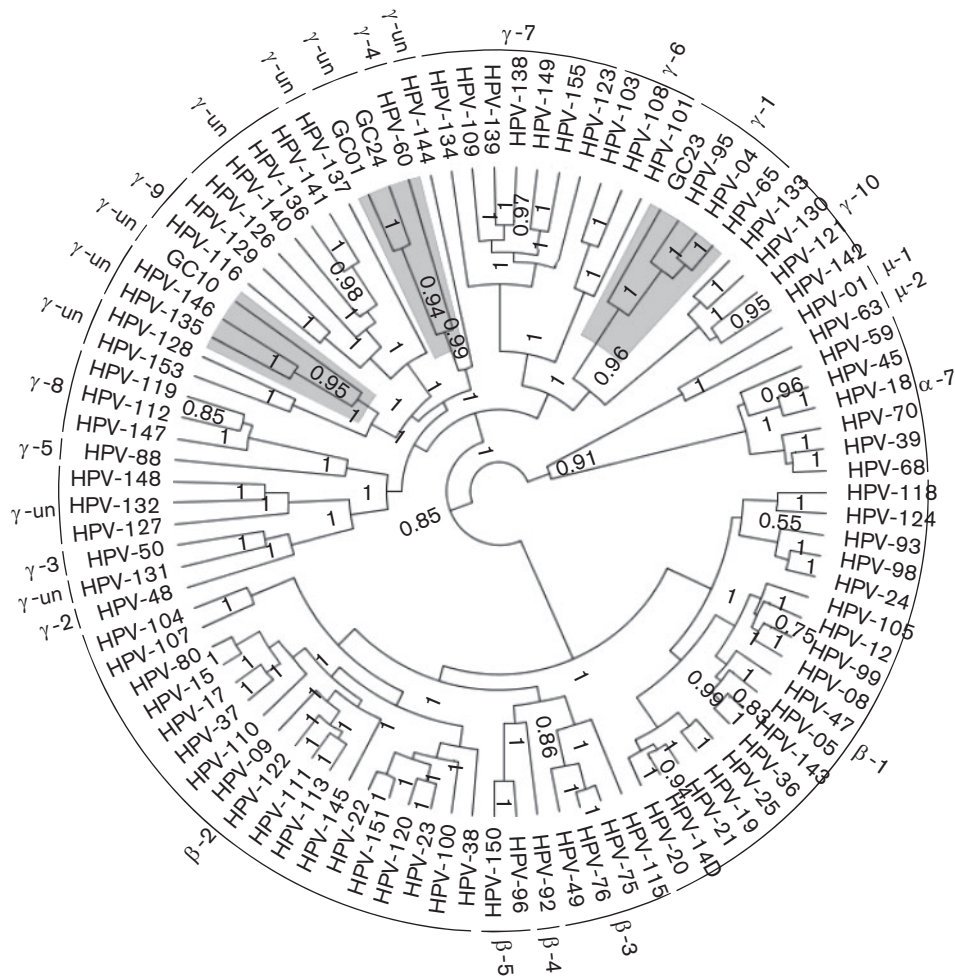‡N. Negative result; P, unidentified 3800–4400 bp amplicon.

Fig. 2. Phylogeny of the putative HPV types found in this study with respect to reference HPVs. Phylogenetic analysis was done of partial L1 ORF sequences from the four putative HPV types identified in this study and reference HPVs from β-, γ- and μ-PV genera. HPVs from α-PV species 7 were used as the outgroup. Node values represent Bayesian posterior probabilities (BPP). Shaded in grey are the GC isolates found in this study with the closest related HPV types. γ-un, Species not included in the current HPV taxonomy (Bernard *et al.*, 2010).

Sequence analysis of the full-length HPV-156 revealed a genome of 7329 bp, with a G + C content of 36.2 mol%. HPV-156 had the principles of genome organization found in PV (Table 3), potentially encoding five early genes (E6, E7, E1, E2 and E4) and two late genes (L2 and L1), as well as a missing E5 ORF, which is a characteristic feature of

Table 2. Pairwise nucleotide identities (%) between putative HPV types and HPV types representing current species within the γ-PV genus (Bernard *et al.*, 2010)

Putative HPV type	γ-PV species									
	1	2	3	4	5	6	7	8	9	10
	HPV-4	HPV-48	HPV-50	HPV-60	HPV-88	HPV-101	HPV-109	HPV-112	HPV-116	HPV-121
GC01	64.6	65.5	64.6	67.1	65.1	63.9	64.1	63.0	65.3	66.7
GC10	65.7	61.1	61.5	63.0	61.2	60.4	67.0	57.5	61.2	61.9
GC23	72.4	65.1	70.0	65.1	64.5	63.6	68.8	62.1	60.2	66.7
GC24	59.5	61.8	63.9	65.3	60.6	57.5	61.5	58.2	61.6	61.9

human β-PV and γ-PV (Köhler *et al.*, 2011). The URR of HPV-156, containing *cis*-responsive elements between the L1 and E6 ORFs, consisted of 544 bp, which falls within the expected length for the γ-PV genus (Köhler *et al.*, 2011). Within the URR, a TATA box (TATAA) was identified, as well as four putative binding sites for E2 [ACC(N)$_6$GGT]. Multiple binding sites for transcriptional regulatory factors such as AP-1, NF-1 and SP-1 were also present within the URR. The putative proteins of the novel HPV type also showed typical domains. The E6 gene product of HPV-156 contained the two characteristic zinc-binding domains CXXC(X)$_{29}$CXXC separated by 36 aa, and three different putative PDZ-binding domains [consensus: X(T/S)X(L/V); HPV-156: PTSL at aa 6–9, KSDL at aa 81–84, and FSLV at aa 122–125] (Fanning & Anderson, 1999). The putative E7 gene product contained one zinc-binding domain, CXXC(X)$_{29}$CXXC, but the LXCXE motif required for binding to the pRB protein was absent. Analysis of the E1-coding sequence showed a typical ATP-binding site of the ATP-dependent helicase [consensus: GXXXXGK(T/S); HPV-156: GPPDTGKS] (Titolo *et al.*, 1999).

Pairwise comparison of the novel L1 ORF confirmed HPV-156 as a γ-PV, showing nucleotide identities ranging from 58.3 to 67.3 % compared with types representing current γ-PV species (Table 3). Therefore, HPV-156 qualified as a novel species within the γ-PV genus according to the current criteria for species definition (de Villiers *et al.*, 2004) (Table 3). The same was true when comparing HPV-156 with the HPV types from the γ-PV genus that have been recently reported and that are awaiting species designation. In fact, pairwise comparisons of the HPV-156 L1 ORF showed nucleotide identities ranging from 60.4 to 64.4 % compared with HPV-126 (61.9 %), HPV-135 (63.6 %), HPV-136 (64.4 %), HPV-137 (60.4 %), HPV-140 (61.8 %), HPV-141 (63.0 %), HPV-144 (64.4 %) and HPV-155 (61.8 %).

HPV-156 showed the highest L1 identity to HPV-60 (67.3 %), member of species γ-4 (Table 3). Although the novel virus was consistently closely related to HPV-60 in both late- and early-gene-derived phylogenies inside the γ-PV genus (Fig. 3), some contradictory relationships were found. In the late-gene analysis (Fig. 3, left), HPV-60 (γ-4) and HPV-156 were the closest relatives of HPV-144 (BPP=0.99). However, in the early-gene analysis, both types were closely related to the unclassified γ-PV types HPV-146, HPV-135, HPV-128 and HPV-153 (BPP=0.83), whilst HPV-144 showed a highly supported relationship (BPP=0.99) with members of γ-1 (Fig. 3, right).

DISCUSSION

In the present study, we designed an approach to facilitate the identification of novel HPV genomes with higher sensitivity than conventional PCR methods used for long DNA fragment amplifications. The hanging-droplet long PCR is based on the use of two generic primer pairs targeting the L1 and E1 gene regions, and the highly sensitive hanging-droplet PCR technique (Walsh *et al.*, 2001) as the amplification strategy.

Table 3. Sizes of putative proteins encoded by the putative ORFs of HPV-156, their nucleotide positions and nucleotide identities with types representing γ-PV species

URR, Upstream regulatory region.

HPV-156 (7329 nt)	Putative ORF							
	E6	E7	E1	E2	E4	L2	L1	URR
Protein size (aa)	140	97	608	399	201	551	509	–
Nucleotide sequence (pre-stop codon) (nt)	106–525	525–815	805–2628	2567–3763	2925–3527	3775–5427	5361–6887	544

| HPV type (species)	Nucleotide identity (%)							
	E6	E7	E1	E2	E4	L2	L1	URR
HPV-4 (γ-1)	58.6	54.9	64.0	54.0	51.7	52.6	61.9	40.4
HPV-48 (γ-2)	57.9	57.4	60.5	56.5	48.4	49.0	62.3	43.0
HPV-50 (γ-3)	54.1	50.0	61.0	55.8	47.2	49.0	62.4	38.1
HPV-60 (γ-4)	59.0	65.3	65.8	60.6	56.6	56.1	67.3	59.2
HPV-88 (γ-5)	54.8	61.2	63.3	55.3	36.0	51.4	64.1	45.0
HPV-101 (γ-6)	–	52.6	57.0	53.7	42.9	47.2	58.3	33.5
HPV-109 (γ-7)	50.1	50.9	60.8	55.3	39.6	49.2	60.7	37.2
HPV-112 (γ-8)	57.9	59.5	61.5	56.9	45.8	50.3	63.2	46.5
HPV116 (γ-9)	57.2	56.4	63.8	55.9	47.4	48.4	60.5	44.4
HPV-121 (γ-10)	54.4	57.1	62.3	56.6	45.4	52.3	61.9	43.8
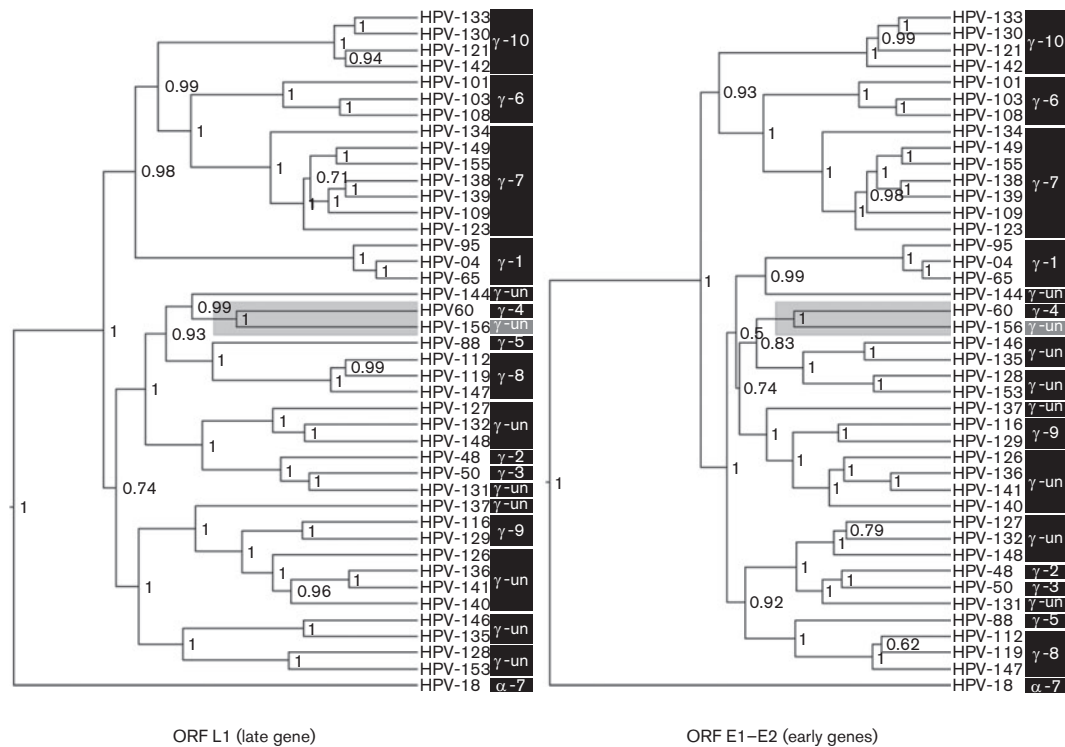
Fig. 3. Early- and late-gene phylogeny of HPV-156, a prototype of a novel species within the γ-PV genus. Phylogenetic trees are shown of HPV-156 and 40 representative types of genus γ-PV based on full-length L1 ORF sequences (left) for late-gene analysis and concatenated E1–E2 ORF sequences (right) for early-gene analysis. In both trees, an α-PV (HPV-18) was used as the outgroup. The numbers on nodes indicate BPP. The closest relative species to HPV-156 are shaded in grey in each tree.

The method was first applied for the amplification of several putative HPV types belonging to the γ-PV genus, using the newly designed γ-PV generic primers from the E1 region and the widely used FAP primers (Forslund *et al.*, 2003). This approach was 100 times more sensitive than a single-round long PCR procedure in the amplification of an HPV-4 genomic half (Fig. 1b). The increase in sensitivity was due to the application of the hanging-droplet PCR technique, used for the first time in this work for the amplification of long DNA fragments. This is clearly an advantage over rolling-circle amplification, which requires the presence of high HPV copy numbers in the sample to succeed in the identification of full-length HPV genomes (Köhler *et al.*, 2010; Köhler *et al.*, 2011). The combination of rolling-circle amplification with long PCR has improved the detection of some virus types (de Villiers and Gunst, 2009; Köhler *et al.*, 2011). However, this still depends on the use of two different methodologies that require individual optimization, and the sensitivity of the whole procedure has not been assessed. As cutaneous HPV types are commonly present at very low copy numbers in both healthy skin and lesions (Vasiljevic *et al.*, 2008; Weissenborn *et al.*, 2005), our strategy is a useful tool to identify novel viruses in these samples.

The hanging-droplet long PCR offered the possibility to screen different viruses in clinical samples with a single methodology, without the need for multiple primer sets to obtain long HPV DNA fragments (Table 1). This is an important advantage over existing methods, as clinical samples are often the only reservoir of the novel viruses under study. Although the generic primer pairs used in this study were not able to amplify the corresponding genomic halves of a given HPV type in a sample (Table 1), we do not exclude the possibility of success with other samples (Fig. 1). It is well known that PCR amplification efficiency depends on the size of the PCR fragment intended to be amplified, the initial amount of target DNA and the number of mismatches between target DNA and primers. The latter seemed to be the cause of the lack of amplification of both genomic halves of the putative HPV types identified in this report. In fact, the GC23 genomic half amplified using the FAP6085-F/E1Gamma-R primer pair presented seven mismatches with the E1Gamma-F/FAP6319-R primer pair (data not shown). The same was true for the 4 kb fragments of putative types GC01, GC10 and GC24, successfully amplified with the E1Gamma-F/FAP6319-R primer pair, but presenting one, four and one mismatches, respectively, with the FAP6085-F/E1Gamma-R primer pair (data not shown). It is worth noting that all primers used for screening were generic; therefore, they could have different efficiencies for each particular type attempting to be amplified. Finally, our

strategy may be used with primers designed for the amplification of 7–8 kb as reported by others (de Villiers & Gunst, 2009; Köhler *et al.*, 2011; Vasiljevic *et al.*, 2008) but with improved sensitivity.

Using the hanging-droplet long PCR, we obtained 4 kb fragments corresponding to genomic halves of four putative HPV types (Table 1). GC01 and GC10 were identified previously as PCR amplicons with the FAP and CUT primers, respectively (Chouhy *et al.*, 2010). However, GC23 and GC24 were novel putative HPV types identified for the first time in this work (Table 1). Moreover, four amplicons of the expected size (termed 'P', Table 1), which must be identified and characterized, were obtained. Curiously, a 4 kb fragment corresponding to HPV-23 (*β*-PV species 2) was identified with the E1Gamma-F/ FAP6319-R primer pair (Table 1). It is worth noting that the generic primers from the E1 gene region were designed for the amplification of HPVs belonging to the *γ*-PV genus. However, the presence of only two mismatches between the E1Gamma-F primer sequence and the corresponding targeting sequence in HPV-23 suggests that, under certain conditions, HPV types belonging to genera other than *γ*-PV may be amplified with this strategy.

Phylogenetic analysis based on partial L1 sequences of the putative HPV types grouped them into the *γ*-PV genus (Fig. 2 and Table 2). The novel GC23 putative HPV type could be assigned to species *γ*-1, whilst GC24 was segregated outside the defined species and was closely related to the GC01 isolate (Fig. 2). Since the GC01 and GC10 isolates were first identified (Chouhy *et al.*, 2010), six new species (*γ*-6 to *γ*-10) have been defined within the *γ*-PV genus (Bernard *et al.*, 2010). However, these isolates cannot be assigned to the current species (Fig. 2), which suggests the need to progress in the identification of the missing taxa in order to have a complete picture of the diversity of this genus.

Most of the HPV types/putative types identified with the hanging-droplet long PCR strategy differed from those that were detected originally with the FAP and/or CUT primer systems (Table 1) (Chouhy *et al.*, 2010). These differences may be due to the particular specificity of each primer pair with respect to the different HPV types/putative types, as the sensitivities of the CUT and FAP amplification methodologies (Chouhy *et al.*, 2010; Forslund *et al.*, 2003) were similar to those obtained with the hanging-droplet long PCR system. All this clearly indicates that the identification of new HPV types depends not only on the use of a highly sensitive technique but also on the combination of different generic primer pairs. Thus, the strategy presented here is versatile and might be applied with different generic primer pairs, targeting the *γ*-PV and other PV genera, to facilitate and speed up characterization of the missing members of the family *Papillomaviridae*. Taking this into account, we started the characterization of one of the putative HPV types, isolate GC01. This novel viral type, designated HPV-156,

presented a genome organization and putative proteins that resembled those of other *γ*-PV (Köhler *et al.*, 2011). The putative E7 and E6 proteins contained one and two zinc-binding domains, respectively, but E7 did not have the pRB-binding motif. Interestingly, three different putative PDZ-binding domains were found within the E6 protein sequence. A specific characteristic of oncogenic mucosal HPV types is a PDZ-binding motif on the carboxyl terminus of E6, which is important in cell proliferation, cell polarity and cell transformation (Cavatorta *et al.*, 2004). However, PDZ-binding domains were not found at the carboxyl terminus of the HPV-156 E6 protein. PDZ domains located outside the carboxyl-terminal region have also been found in other HPV types belonging to the *γ*-PV genus such as HPV-130, HPV-131, HPV-132, HPV-133, HPV-134 and HPV-149 (Köhler *et al.*, 2011).

HPV-156 was first identified as the GC01 putative HPV type with FAP primers in a sun-exposed healthy skin sample derived from an 83-year-old male (Table 1, patient 82) with basal cell carcinoma in the upper lip (Chouhy *et al.*, 2010). In the present study, its full-length genome was also characterized from a healthy skin sample (Table 1, patient 89), suggesting HPV-156 as a commensal agent. Further detection of HPV-156 in lesions and healthy skin in individuals from other geographical areas may clarify the medical significance of this virus and its global prevalence.

Phylogenetic analyses of the complete L1 ORF of the novel virus and types representing current species indicated HPV-156 as the prototype of a new species within the *γ*-PV genus (Fig. 3, left, and Table 3). HPV-156 also meets the criteria for a new species with respect to the recently reported *γ*-PV types with unclassified species (HPV-126, HPV-135, HPV-136, HPV-137, HPV-140, HPV-141, HPV-144 and HPV-155; http://pave.niaid.nih.gov).

We investigated early and late genes in separate phylogenetic analyses to explore possible incongruent tree topologies. Although HPV-156 and HPV-60 (*γ*-4) were found to be the closest relatives in both phylogenies without incongruence between them (Fig. 3), the internal topologies showed contradictory relationships. Incongruent tree topologies between early- and late-gene phylogenies have been explained by recombination events (Gottschling *et al.*, 2007), which have been detected in *α*-PV (Narechania *et al.*, 2005), *γ*-PV (Köhler *et al.*, 2011), o-PV and *υ*-PV (Rector *et al.*, 2008; Robles-Sikisaka *et al.*, 2012) genera. However, general conclusions about PV evolution may be premature and largely speculative as long as the knowledge about PV diversity is limited (Bravo *et al.*, 2010). Increasing the taxon sampling by isolating and sequencing novel PVs may shed light on virus evolution and on the interactions with their hosts. One of the cornerstones for the identification of new full-length PV types is the possibility of amplifying them with high sensitivity. In this regard, our report provides a versatile and highly sensitive approach that allowed the identification not only of a new HPV but also of a new species within the *γ*-PV genus. The application of the

hanging-droplet long PCR system with primers targeting the different genera in which both human and non-human PVs are distributed will help us to gain a complete picture of the family *Papillomaviridae* to elucidate the diversity, evolution and medical implications of these viruses.

METHODS

Skin samples. Nineteen skin samples from 17 immunocompetent patients were used for this study. All samples were collected in a previous study to determine the HPV prevalence and type spectrum in skin diseases and in healthy skin areas in a group of patients from Argentina (Chouhy *et al.*, 2010). All samples selected contained one or more putative HPV types from the γ-PV genus according to the CUT and/or FAP primer systems screening (Chouhy *et al.*, 2010).

γ-PV generic primer design in the E1 gene region. DNA sequences from the E1 ORF of eight characterized HPVs belonging to six known species within the γ-PV genus [HPV-4 and HPV-65 (γ-1), HPV-48 (γ-2), HPV-50 (γ-3), HPV-60 (γ-4), HPV-88 (γ-5), HPV-101 and HPV-103 (γ-6)] were aligned using MEGA4 (Tamura *et al.*, 2007). Primer candidates obtained with the Prima-Clade program (http://www.umsl.edu/services/kellogg) were compared to find those presenting fewer mismatches, and analysed for primer–dimer and primer-hairpin formations. After this analysis, E1Gamma-F (5′-KGGHCCWCC-AGATACWGG-3′) and E1Gamma-R (5′-TCTTGIWGTGCAWCGR-AACG-3′) primers were selected, corresponding to positions 2093–2110 and 2572–2591, respectively, in the HPV-4 genome.

'Hanging-droplet' long PCR. The general strategy for generating HPV DNA genomic halves is depicted in Fig. 1(a). E1 γ-PV primers were combined with the previously described FAP primers (Forslund *et al.*, 2003) to generate overlapping amplicons with expected lengths of 3723 bp (E1Gamma-F/FAP6319-R primer pair) and 4362 bp (E1Gamma-R/FAP6085-F primer pair) in the HPV-4 genome (GenBank accession no. NC_001457). Amplifications were performed using the single-tube hanging-droplet PCR technique (Walsh *et al.*, 2001), with the conditions described below. A thermal cycler (Mastercycler Personal; Eppendorf) was used in all experiments involving PCR.

The primary 20 µl reaction mixture was placed in a reaction tube and covered with one drop of mineral oil. After the addition of the sample (5 µl), the mixture had a final volume of 25 µl containing 0.5 µM of each combination of the generic primers (E1Gamma-F/FAP6319-R or FAP6085-F/ E1Gamma-R), 500 µM of each dNTP, 1× PCR buffer and 2 U enzyme mix (Expand Long Range dNTPack; Roche). Before the tube was closed, a 25 µl droplet, containing the same reaction mixture (but with 1.8 µM of each combination of E1Gamma-F/ FAP6319-R or FAP6085-F/E1Gamma-R generic primers, 600 µM of each dNTP and 4 U of enzyme mix), was placed in the centre of the inside of the reaction tube cap. Using the thermal cycler programmed for block temperature without heated lid, the mixture was heated for 2 min at 92 °C, followed by 25 cycles of 15 s at 92 °C, 48 °C (E1Gamma-F/FAP6319-R) or 50 °C (FAP6085-F/E1Gamma-R) for 40 s and 4 min at 68 °C. After the first round of amplification, the 'hanging droplet' was incorporated into the reaction mixture (final volume 50 µl) by spinning down at 11 000 **g** for 1 min, and a second round of 40 cycles was performed [15 s at 92 °C, 48 °C (E1Gamma-F/ FAP6319-R) or 50 °C (FAP6085-F/E1Gamma-R) for 40 s and 4 min at 68 °C (in the last 30 cycles the elongation time was increased by 15 s each cycle)]. HPV amplicons were identified by size determination under UV light after electrophoresis in a 1 % agarose gel and ethidium bromide staining.

All hanging-droplet long PCR optimizations were carried out with a plasmid containing the complete genome of HPV-4 as template, available in our laboratory. The sensitivity of the new method was compared with an optimized protocol using simple long PCR with the E1Gamma-R/FAP6085-F primer pair. The reaction mixture (final volume 50 µl) and the amplification protocol was the same as that used in the amplification of the second round of the hanging-droplet long PCR.

Cloning, DNA sequencing and phylogenetic analysis. Bands of ~4 kb were excised from the gel and purified using a NucleoSpin Extract II kit (Macherey-Nagel). All the amplicons were cloned using a pGEM-T Easy Cloning kit (Promega). DNA sequencing was performed using sequencing facilities at the University of Maine DNA Sequencing Facility (ME, USA).

Sequences were compared with available HPV sequences in GenBank (http://www.ncbi.nlm.nih.gov/) using the BLAST server. Protein sequence-derived nucleotide multiple alignments and pairwise alignments were performed with MEGA5 (Tamura *et al.*, 2011), and phylogenetic relationships were inferred by Bayesian analysis using BEAST version 1.6.2 (Drummond & Rambaut, 2007). To do this, Markov chain Monte Carlo simulations were performed during 10^7 generations, sampling one state every 1000 generations, with a burn-in of 10 %. The evolutionary substitution model for each run was set as rtREV + γ.

For the phylogenetic analysis of novel (GC23 and GC24) and previously identified (GC01 and GC10) putative HPV types, the corresponding FAP amplicon and L1 sequences from representative HPV types were used. New putative HPV types and novel putative species within a genus were defined according to the current criteria based on the nucleotide identities in the L1 fragment sequences (de Villiers *et al.*, 2004).

For the phylogenetic analysis of HPV-156, sequences from representative HPV types of the γ-PV genus were used. In order to explore possible incongruent tree topologies of HPV-156, we investigated early and late genes in separate phylogenetic analyses. For late-gene analysis, the L1 ORF was used. For early-gene analyses, the E1–E2 ORFs were used, but the hinge region of E2, including the highly divergent E4 gene, was eliminated from all phylogenetic analyses. All sequences from the previously characterized HPV types were obtained from the PV genome database (http://pave.niaid.nih.gov).

Identification of the full-length genome of HPV-156. HPV-156 was isolated from a swab sample collected from a sun-exposed healthy skin area derived from a 60-year-old female with a seborrheic keratosis lesion on the face. HPV-156 corresponded to the previously reported putative type GC01 (Chouhy *et al.*, 2010). The complete genome sequence of the novel HPV-156 type was obtained by generating overlapping amplicons. One of these was obtained with the E1Gamma-F/FAP6319-R primer pair and the hanging-droplet long PCR method described above. The other overlapping amplicon was obtained with the specific primers GC01-F (5′-CCTTATGGATGCAGACCACTGG-3′) and GC01-R (5′-AACTCCAGCAAGCAAAGGTAGCA-3′), both designed using the 3′ and 5′ regions of the GC01 genomic half obtained with the E1Gamma-F/FAP6319-R primer pair as sequence template and the FastPCR program (Kalendar *et al.*, 2009). Reaction mixtures and cycling conditions were the same as those used in the hanging-droplet long PCR, except in the reaction mix of the second round of amplification in which 1.2 µM each specific primer was used. The annealing temperatures of the GC01-F/GC01-R primer pair were 52 and 50 °C in the first and second rounds of amplification, respectively. Amplicons of ~4 kb were purified, cloned and sequenced as described above. Each GC01 genomic half was fully sequenced by primer walking on both DNA strands for complete identification. DNA clones and the corresponding sequences were submitted to the International

Reference Centre for Papillomaviruses at the German Cancer Research Centre (Heidelberg, Germany) for official designation as HPV-156, and the sequences were reconfirmed.

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