# Betapapillomavirus infections : natural history and disease association

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CHAPTER 3

# Betapapillomaviruses frequently persist in the skin of healthy individuals

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# Betapapillomaviruses frequently persist in the skin of healthy individuals

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Infections with human papillomaviruses (HPVs) belonging to the genus *Betapapillomavirus* have been linked to the development of non-melanoma skin cancer. Although persistence is expected, systematic investigation of this aspect of betapapillomavirus ($\beta$-PV) infection has not been conducted. This study investigated the prevalence and persistence of 25 known $\beta$-PV types in the skin of immunocompetent individuals. Over a 2 year period, eight consecutive plucked eyebrow hair samples taken from 23 healthy individuals were analysed for the presence of $\beta$-PV DNA. Using a recently published general $\beta$-PV PCR and genotyping method, 61 % of the individuals were $\beta$-PV DNA positive for one or more types at intake, whereas during follow-up this percentage rose to 96 %. HPV23 was the most frequently detected $\beta$-PV type. Type-specific $\beta$-PV DNA was detected over 6 months or longer in 74 % of the individuals. In 57 % of the individuals, DNA from multiple $\beta$-PV types was detected simultaneously for 6 months or longer. When the detection intervals of all $\beta$-PV type-specific infections in the study population were considered, a substantial proportion, 48 %, lasted at least half a year. The consistent $\beta$-PV patterns found over time in most individuals strongly suggested that $\beta$-PV DNA detection in plucked eyebrow hairs reveals true $\beta$-PV infection. If the minimum interval of detection was set at 6 months, persistent $\beta$-PV infections were found in the majority of the study population (74 %).

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## INTRODUCTION

The human papillomavirus (HPV) types initially found in non-melanoma skin cancers [basal cell carcinomas and squamous cell carcinomas (SCCs)] of epidermodysplasia verruciformis (EV) patients, the so-called EV-HPV types, belong to the genus *Betapapillomavirus* (de Villiers *et al.*, 2004). At present, 25 betapapillomavirus ($\beta$-PV) types (HPV types 5, 8, 9, 12, 14, 15, 17, 19–25, 36–38, 47, 49, 75, 76, 80, cand92, 93 and cand96) have been fully sequenced. Based on partial sequences, probably more than 35 new types have to be added to this list (Pfister, 2003).

$\beta$-PVs are frequently detected in non-melanoma skin cancers in both immunocompetent and immunosuppressed individuals (Harwood & Proby, 2002; Pfister, 2003; Sterling, 2005). Although $\beta$-PVs are also prevalent in swabs, plucked hairs and biopsies from normal skin (Antonsson *et al.*, 2003a; Boxman *et al.*, 1997; Forslund *et al.*, 2003; de Oliveira *et al.*, 2004), a number of studies have now shown statistically significant associations between markers of $\beta$-PV infection and the development of SCC (Boxman *et al.*, 2000, 2001; Feltkamp *et al.*, 2003; Harwood *et al.*, 2004; Struijk *et al.*, 2003, 2006). Based on experimental data, it is expected that $\beta$-PVs play a role early in cancer development, with less of a role in maintenance of the transformed state (Pfister, 2003). The ability of the viral E6 protein to inhibit ultraviolet (UV) B-induced apoptosis (Jackson & Storey, 2000; Jackson *et al.*, 2000) and DNA repair (Giampieri & Storey, 2004; Iftner *et al.*, 2002; Jackson & Storey, 2000; Jackson *et al.*, 2000), as demonstrated for instance for HPV5, may represent such an early mechanism of transformation by a $\beta$-PV. As a result, infected keratinocytes with UV-induced DNA damage and mutations may accumulate in the epidermis, bringing the risk of genomic instability and oncogenic transformation (Bouwes Bavinck & Feltkamp, 2004). It is expected that the chance of developing $\beta$-PV-associated skin cancer through such a mechanism would increase with the duration of the $\beta$-PV infection.

Long-lasting, persistent infections of the cervix with mucosal HPV types have been described regularly (Cuschieri *et al.*, 2005; Harper *et al.*, 2006; Ho *et al.*,

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1998; Moscicki *et al.*, 2001; Schlecht *et al.*, 2001). It is generally accepted that women with persistent high-risk genital HPV infections are at a relatively high risk of developing (pre)malignant cervical lesions (Schlecht *et al.*, 2001). In contrast to mucosal HPV types, very little is known about the natural history of cutaneous infections with $\beta$-PV types. Beta-PV infections probably occur very early in life, starting immediately after birth (Antonsson *et al.*, 2003b). Lesional skin of renal transplant recipients who frequently develop benign and (pre)malignant squamous skin lesions consistently contains one or more $\beta$-PV types (Berkhout *et al.*, 2000). A more recent study has been published that found cutaneous HPV types persisting in forehead swab samples (Hazard *et al.*, 2007).

In order to study the duration of cutaneous $\beta$-PV infections in healthy individuals, we monitored the presence of DNA from 25 $\beta$-PV types over a 2 year period in plucked eyebrow hairs collected from a cohort of healthy adults. Previously, $\beta$-PV DNA has been found in plucked hairs from different sites of the human body, revealing the hair bulb as a possible reservoir of HPV types belonging to the $\beta$-PV genus (Boxman *et al.*, 1997). The systemic approach of collecting eyebrow hair samples over time enabled us at the same time to estimate the value of detecting $\beta$-PV DNA in a single plucked hair sample with respect to predicting a person's $\beta$-PV infection status. This information is particularly relevant in evaluating results from past and future epidemiological studies, which often make use of plucked hair samples to determine $\beta$-PV infection.

## METHODS

**Study population.** Twenty-three healthy Dutch adult volunteers were recruited. None of the individuals was anamnestically known to have or have had skin cancer. All participants gave signed informed consent and the study was approved by the local medical ethical commission.

Eight to ten eyebrow hairs were plucked at every time point over a total period of 2 years. For the first 6 months, eyebrow hair samples were obtained monthly and thereafter 1 and 2 years after the first time point. No individuals were lost during follow-up.

**Sample collection.** Hair sampling was performed by taking eight to ten eyebrow hairs, four to five each from the left and the right eyebrow, from each subject with a clean pair of stainless steel tweezers while wearing clean rubber gloves. Between individuals, the tweezers were cleaned either with a 0.5 % sodium hypochlorite solution or with a 0.1 M HCl solution and subsequently with distilled water or with 70 % ethanol to avoid contamination. Special care was taken to check that all plucked hairs had a hair bulb attached. The samples were kept at −20 °C in Eppendorf tubes with screw caps with external threading to prevent loss of material and contamination due to hairs getting stuck in the thread.

From one subject, ten additional single hairs were plucked at the first time point, five from the left and five from the right eyebrow. Between the plucking of each hair, the tweezers were cleaned as described above.

**DNA isolation.** DNA isolations were carried out using a QIAamp DNA mini kit (Qiagen). Briefly, hairs were pre-treated overnight with proteinase K solution according to the manufacturer's instructions. After lysis with 200 µl buffer AL, half of the volume was stored at −70 °C whilst the other half was processed further according to the manufacturer's instructions. To monitor for contamination, after each tenth sample a negative isolation control was included that was processed in parallel with the other samples. These isolation controls tested negative in all cases.

**PCR and hybridization.** $\beta$-PV detection and genotyping was carried out as described previously (de Koning *et al.*, 2006). Briefly, PM-PCR was performed in a final reaction volume of 50 µl, containing 10 µl of the isolated DNA, 2.5 mM $MgCl_2$, 1× GeneAmp PCR Buffer II, 0.2 mM deoxynucleoside triphosphates, 1.5 U AmpliTaq Gold DNA polymerase and 10 µl of the PM primer mix. The PCR was performed by a 9 min pre-heating step at 94 °C, followed by 35 cycles of amplification comprising 30 s at 94 °C, 45 s at 52 °C and 45 s at 72 °C. The PCR was ended by a final elongation step of 5 min at 72 °C. As a positive PCR control, a $\beta$-PV plasmid clone was included at an amount approximately 100 times the limit of detection of the assay. The positive PCR control was detected in all cases. The quality of the isolated DNA samples was checked by amplifying a 268 bp fragment from the $\beta$-globin gene with primers PC04 and GH20 (Saiki *et al.*, 1988).

All amplimers were subsequently analysed with a reverse hybridization assay (RHA) that permitted specific detection and identification of the 25 established $\beta$-PV genotypes (i.e. types 5, 8, 9, 12, 14, 15, 17, 19–25, 36–38, 47, 49, 75, 76, 80, cand92, 93 and cand96). The RHA was performed according to the manufacturer's instructions (Diassay BV). Briefly, 10 µl of the biotin-labelled amplimer was mixed with 10 µl denaturation solution and 10 µl 3B buffer in a plastic trough containing the $\beta$-PV strip. The mix was incubated for 5 min at room temperature. Two millilitres of pre-warmed (37 °C) hybridization buffer (3× SSC, 0.1 % SDS) was added and incubated at 50±0.5 °C for 1 h. All incubations and washing steps were performed automatically in an Auto-LiPA (Tecan GmbH). The strips were washed twice for 30 s and once for 30 min at 50 °C with 2 ml hybridization solution. Following this stringent wash, the strips were incubated with 2 ml alkaline phosphatase–streptavidin conjugate for 30 min at room temperature. Strips were washed twice with 2 ml rinse solution and once with 2 ml substrate buffer. Two millilitres of substrate (5-bromo-4-chloro-3-indolylphosphate and nitro blue tetrazolium) were added and incubated for 30 min at room temperature. The reaction was stopped by washing for 3 and 10 min with rinse solution followed by a wash with 2 ml of water. The strips were dried and the purple-coloured bands were visually scored and interpreted.

## RESULTS

### Detection of $\beta$-PV

Eyebrow hair samples of 23 healthy individuals were tested in this study. The mean age at the start of the study was 35 years, ranging from 21 to 64 years. The number of males and females was comparable (11 and 12, respectively). The inconvenience of having ten eyebrow hairs plucked at each visit was generally considered to be insignificant. All samples were positive by $\beta$-globin PCR, confirming the presence of amplifiable DNA.

Except for one person (individual 12, Table 1), all individuals were $\beta$-PV DNA positive at one or more time points during the follow-up (22/23 individuals, 96 %). In

**Table 1.** Overview of HPV types detected in a total of 184 eyebrow hair samples, collected from 23 individuals at eight time points over a study period of 2 years

The numbers represent the HPV type(s) detected in the tested hair samples. All tested HPV types belonged to the genus *Betapapillomavirus*.

| Individual | Month | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 12 | 24 |
| 1 | 23, 36 | 8, 23, 93 | 14, 23, 93 | 8 | 23, 93 | 8 | 22, 93 | 22 |
| 2 | – | – | – | 19 | 19 | 19 | – | 19 |
| 3 | 5, 23, 36, 93 | 5, 17, 23, 93 | 5, 23, 93 | 23, 93 | 23, 38 | 5, 23, 93 | 23 | 5, 23, 36, 93 |
| 4 | 5, 23, 38, 76 | 8, 15, 23, 38 | 23, 38 | 15, 38 | 15, 38 | 38 | – | 23, 38, 93 |
| 5* | 8, 15, 20, 23, 38, 49, 92 | 8, 15, 20, 23, 38, 92 | 8, 9, 15, 20, 23, 38, 49, 92 | 8, 9, 15, 20, 23, 38, 92 | 8, 15, 20, 23, 38, 92 | 8, 9, 15, 17, 20, 23, 36, 38, 92 | 8, 15, 20, 23, 38, 92 | 8, 9, 15, 17, 20, 38, 92 |
| 6 | – | 15 | – | – | 23, 37 | 15, 23 | – | 15, 23 |
| 7 | 12, 38 | 12, 38 | – | 17 | 80 | 15, 80 | 5 | – |
| 8 | – | 8, 23 | 23 | – | – | 23 | – | 8, 14, 23 |
| 9 | 23, 80 | 80 | 23, 80 | 23, 80 | 24, 80 | 23, 38, 80 | 23, 80 | 23 |
| 10 | 8, 23, 24, 92 | 8, 23, 24, 75, 80, 92 | 8, 23, 24, 75, 93 | 8, 24 | 8, 23 | 8, 23, 24, 75 | 24 | 36, 75, 93 |
| 11 | 15 | 8, 23, 38 | 23, 38 | 5, 23 | 38 | 23, 38 | 14, 23 | 23, 96 |
| 12 | – | – | – | – | – | – | – | – |
| 13 | 15, 23 | 23 | 23 | – | 75 | 15, 23 | 15, 23, 76 | 23 |
| 14 | – | 25 | 25 | 25 | 25 | – | – | – |
| 15 | 38 | 24, 93 | 23, 24, 93 | 23, 38, 93 | 24, 93 | 93 | – | 12, 22, 24, 38, 93 |
| 16 | – | 38 | – | 8, 38 | – | 8 | – | 20 |
| 17 | 12, 23, 24, 80 | 12, 17, 24, 80 | 12, 23, 24, 80 | 12, 23, 24, 80 | 12, 23, 24, 80 | 12, 23, 24, 80 | 12, 23, 24 | 12, 17, 23, 24, 80 |
| 18 | – | – | 23 | – | – | 23 | – | – |
| 19 | 5, 20, 23 | 5, 20 | 5, 20, 23 | 5, 20 | 5, 20, 23, 80 | 5, 20 | 5, 20, 23 | 5, 20 |
| 20 | – | – | 9 | 23 | – | 9 | 23 | 23 |
| 21 | 23 | – | – | – | – | 23, 36 | – | 17, 36 |
| 22 | – | – | 8 | – | – | – | – | 24, 36, 75 |
| 23 | 5, 8, 15, 23, 25, 36, 37 | 5, 15, 23, 25, 36, 37 | 5, 15, 23, 25, 36, 37 | 5, 15, 23, 25, 36, 37 | 5, 15, 25, 36, 37 | 5, 15, 25, 37 | 5, 15, 23, 25, 36, 37 | 5, 15, 23, 25, 36, 37 |

*Ten additional single hairs from this individual were taken at month 0 and were analysed separately. Results are shown in Table 2.

most individuals (19/23, 83 %), $\beta$-PV DNA was detected in at least four of the eight hair samples. Nine of the 23 individuals (39 %) were HPV positive at all time points (Table 1). HPV types 5 and 8, possibly being high-risk cutaneous HPV types (Cogliano *et al.*, 2005), were detected in 6/23 (26 %) and 9/23 (39 %) of the individuals, respectively. Except for HPV21 and HPV47, DNA from all tested $\beta$-PV types was present. HPV23 was the most frequently found type, with 17/23 individuals (74 %) being HPV23 positive at some time point (Table 1).

In the majority of individuals (18/23, 78 %), DNA from different $\beta$-PV types was simultaneously detected at any time point, and a high multiplicity of HPV types was seen, as five or more $\beta$-PV types were present at one or more time points in 43 % (10/23) of the tested individuals (Table 1). A maximum of ten different $\beta$-PV types was found in one individual (individual 5, Table 1) over the whole period, with a maximum of nine $\beta$-PV types in a single sample at month 5.

## Detection of $\beta$-PV in single hairs

To investigate the distribution of HPV types in single hairs and to estimate the contribution of a single hair to the $\beta$-PV profile obtained from a hair sample made up of ten hairs, ten additional single eyebrow hairs were plucked at month 0 from individual 5 (Table 1). In nine out of ten single hairs, $\beta$-PV DNA was detected (Table 2). All of the $\beta$-PV-positive single hairs contained multiple HPV types, ranging from two to eight. In comparison with analysis of the samples consisting of ten hairs (Table 1), the median number of HPV types found in the single hairs (six types) was almost identical to that calculated from the regular hair samples (seven types). However, in the single hair samples,

**Table 2.** Overview of the HPV types detected in the ten additional single hairs taken from individual 5 at month 0

| Hair sample | HPV types detected |
|---|---|
| Complete hair sample* | 8, 15, 20, 23, 38, 49, 92 |
| Left 1 | 8, 17, 20, 23, 38, 49, 92 |
| Left 2 | 8, 12, 14, 17, 20, 23, 38, 49 |
| Left 3 | 8, 19, 23, 38, 49 |
| Left 4 | 8, 17, 19, 20, 23, 38, 49 |
| Left 5 | 12, 17, 19, 20, 23, 49, 92 |
| Right 1 | – |
| Right 2 | 19, 20, 23, 38, 92 |
| Right 3 | 12, 19, 23 |
| Right 4 | 19, 49 |
| Right 5 | 8, 9, 20, 23, 38, 49, 92 |

*The complete hair sample, made up of ten other plucked eyebrow hairs, was identical to that shown for individual 5 at month 0 in Table 1.

five additional β-PV types were observed compared with the regular hair sample at month 0. In contrast, HPV15 was present in the regular hair sample but not detected in the single hairs.

### Detection of β-PV infection over time

Next, the duration of β-PV infection was investigated. To this end, the intervals of detection of the specific β-PV types were calculated by taking the two time points furthest apart that were positive for that type. Because of possible false negatives (see Discussion), it was not necessary that all time points in between the PV type-positive time points furthest apart were positive for that type. However, in 25 out of the 65 times (38 %) of repetitive β-PV detection, each time point in between was positive for that particular β-PV type.

Table 3 shows the number of times that a specific β-PV type was detected within an individual over a specific time interval (0, 1–5 months or ⩾6 months). These numbers were scored for all β-PV types together (last line in Table 3) and are shown for every time period in Fig. 1. In 36 instances, a β-PV type was found only once in an individual (0 month time interval, Table 3 and Fig. 1). In 17 instances, a β-PV type was present over 1–5 months in 11/23 (48 %) individuals, whereas there were 48 instances where a β-PV type was found over a period of at least 6 months in 17/23 individuals (74 %). Twenty-two per cent of the individuals (5/23) had HPV type(s) that were detected in all eight consecutive hair samples during the 2 year follow-up (Table 1).

### Value of β-PV detection

Finally, on the basis of the data presented in Table 3, we estimated the chance that detection of a β-PV type in one hair sample represents long-term infection. To estimate this chance, we added together the number of times that an

**Table 3.** Schematic overview depicting the number of times that a specific HPV type was detected within an individual at the indicated time intervals

For instance, HPV8 was detected in a total of nine individuals (any time), in four individuals only once (0 month), in three individuals at the most 5 months apart (1–5 months) and in two individuals at least 6 months apart (⩾6 months).

| HPV type | Time interval (months) | | | Any time |
|---|---|---|---|---|
| | 0 | 1–5 | ⩾6 | |
| 5 | 3 | 0 | 3 | 6 |
| 8 | 4 | 3 | 2 | 9 |
| 9 | 0 | 1 | 1 | 2 |
| 12 | 1 | 1 | 1 | 3 |
| 14 | 3 | 0 | 0 | 3 |
| 15 | 2 | 1 | 4 | 7 |
| 17 | 3 | 0 | 2 | 5 |
| 19 | 0 | 0 | 1 | 1 |
| 20 | 1 | 0 | 2 | 3 |
| 21 | 0 | 0 | 0 | 0 |
| 22 | 1 | 0 | 1 | 2 |
| 23 | 1 | 4 | 12 | 17 |
| 24 | 2 | 0 | 3 | 5 |
| 25 | 0 | 1 | 1 | 2 |
| 36 | 4 | 0 | 3 | 7 |
| 37 | 1 | 0 | 1 | 2 |
| 38 | 2 | 3 | 3 | 8 |
| 47 | 0 | 0 | 0 | 0 |
| 49 | 0 | 1 | 0 | 1 |
| 75 | 2 | 0 | 1 | 3 |
| 76 | 2 | 0 | 0 | 2 |
| 80 | 2 | 1 | 2 | 5 |
| 92 | 0 | 1 | 1 | 2 |
| 93 | 1 | 0 | 4 | 5 |
| 96 | 1 | 0 | 0 | 1 |
| Any type* | 36 | 17 | 48 | 101 |

*As the majority of affected individuals harboured multiple HPV types, the total number of times in which any β-PV type was detected within an individual ($n$=101) exceeds the number of participants in the study ($n$=23).

HPV type was detected only once in an individual (36 times), the number of times that an HPV type was present during 1–5 months (17 times) and the number of times that an HPV type was found for ⩾6 months (48 times). When the total number of β-PV type-specific detections was regarded (36 + 17 + 48 = 101), two-thirds originated from detection lasting for at least 1 month [(17 + 48)/101, 64 %] and almost half originated from detection lasting for at least 6 months (48/101, 48 %) (Table 3).

## DISCUSSION

This study systematically investigated the presence of β-PV DNA in plucked hairs from healthy individuals over time.

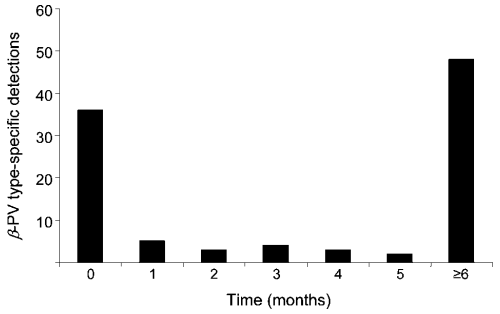


**Fig. 1.** Distribution of type-specific $\beta$-PV detection over time. The bars indicate the number of times that a $\beta$-PV type was detected within an individual over the indicated time interval. As the majority of affected individuals harboured multiple HPV types, the total number of detections exceeds the number of participants in the study.

Despite its small size, this cohort study offered the unique possibility of finding evidence of persistent $\beta$-PV infection and of evaluating the use of plucked hair samples to determine a person's $\beta$-PV infection status.

A substantial number of individuals (21/23) tested positive for particular HPV types at multiple visits during the study. The most likely explanation for this finding is underlying persistent $\beta$-PV infection with the HPV type(s) in question. The consistency of our findings over a 2 year time period makes natural HPV contamination (detection of passenger HPV types) unlikely, although this cannot be formally excluded. In this regard, it is worthwhile mentioning that a small subset of the tested individuals (nos 2, 4, 6, 8 and 10; Table 1) shared a student household, at least at some period during the follow-up (range 6–12 months). Despite the fact that they could have picked up 'contaminating' $\beta$-PV from their environment, these participants displayed unique HPV profiles. This observation argues against HPV contamination as a main source of HPV detected in the plucked eyebrow hairs. Furthermore, it suggests that the infectivity of $\beta$-PV should be considered to be low in such a household setting or, alternatively, that the majority of available sites of infection are already taken by the $\beta$-PVs that make up a personal HPV profile.

To monitor the presence of $\beta$-PV DNA in the plucked eyebrow hair samples, we used a sensitive and broadly reactive PCR specific for HPV types belonging to the $\beta$-PV genus combined with a reverse hybridization method. Both were recently developed and described (de Koning *et al.*, 2006). In total, 23 of the 25 known $\beta$-PV types were detected in this study. HPV types 21 and 47 were not found. The analytical sensitivity of the primer set for each of the 25 $\beta$-PV types is between 10 and 100 copies per PCR (de Koning *et al.*, 2006). It should be noted that in 68 % of the $\beta$-PV-positive samples, multiple types were found. Competition among different genotypes and the preferential use of a subset of PCR primers in the mix of broad-spectrum primers could play an important role. If one HPV genotype is present in high molar excess over the other, the minor genotype could be out-competed and remain unidentified (van Doorn *et al.*, 2006). This phenomenon is a common problem in broad-spectrum PCRs and can lead to an underestimation of the number of HPV genotypes within the same sample.

Only one of the 23 individuals remained negative for $\beta$-PV DNA throughout the study, strengthening earlier data that showed a high prevalence of $\beta$-PV types in plucked eyebrow hairs (Antonsson *et al.*, 2000; Boxman *et al.*, 1997; Struijk *et al.*, 2006). Whether the hair follicle is indeed a reservoir for $\beta$-PV types remains unknown. At present, it cannot be excluded that $\beta$-PVs are primarily present in keratinocytes surrounding the hair bulb that are removed simultaneously with plucking of the hair. Experimental studies using *in situ* hybridization or microdissection should clarify this issue by demonstrating the local presence of viral nucleic acid.

In the field of mucosal HPV research, different definitions are used for persistent infections (Cuschieri *et al.*, 2005; Harper *et al.*, 2006; Ho *et al.*, 1998; Moscicki *et al.*, 2001; Schlecht *et al.*, 2001). In this study, different time intervals were used as the criterion for persistence. If the $\geqslant$6 month interval was applied, 48 $\beta$-PV type-specific persistent infections were observed in 17 individuals (74 %). When the interval was extended to $\geqslant$12 or $\geqslant$18 months, only a slight decrease in persistent infections was seen to 42 and 41 persistent infections in 17 and 16 individuals, respectively. The percentage of individuals persistently infected with multiple HPV types ranged from 57 % ($\geqslant$6 month interval) to 43 % and 43 % ($\geqslant$12 and $\geqslant$18 month interval, respectively). Taken together, our data strongly suggest that (multiple) persistent infections occur in a large percentage of the healthy population.

The duration of persistence of a specific $\beta$-PV type was calculated by taking the two time points furthest apart that were positive for that type, without requiring that all time points in between were also positive for that type. This is in contrast to some studies on mucosal HPV persistence (Cuschieri *et al.*, 2005; Harper *et al.*, 2006; Ho *et al.*, 1998; Moscicki *et al.*, 2001; Schlecht *et al.*, 2001). In these studies, a negative HPV test following a positive test was considered to indicate resolution of the HPV infection. It should be noted, however, that intermittent time point(s) negative for the respective $\beta$-PV type(s) could also be due to competition or sample heterogeneity. The negative effect of sample heterogeneity on $\beta$-PV detection has to be considered, as the $\beta$-PV loads measured in a subset of the plucked hair samples was always less than one copy per cell (data not shown).

In addition to studying the natural history of $\beta$-PV infections in healthy individuals, this study offered the opportunity to verify the use of $\beta$-PV DNA detection in

eyebrow hairs as a reliable and reproducible method of establishing β-PV infection. This is especially relevant in view of screening used to determine a person's β-PV status in epidemiological studies (Boxman *et al.*, 2000, 2001; Struijk *et al.*, 2003, 2006). For this aim, we determined the value of screening for β-PVs by estimating the chance that the detected β-PV types represented persistent infection. The calculations based on the data presented in Table 3 showed that two-thirds of the β-PV types detected in our study represented an infection lasting $\geqslant$1 month and almost half represented a persistent infection of at least 6 months. It should be noticed that infections could have been present before the start of the study and that therefore their duration may have been underestimated. Taken together, these findings seem to indicate that β-PV DNA detection and genotyping in a sample consisting of ten plucked eyebrow hairs is an easy and valid method for determining β-PV infections in epidemiological studies.

The use of hair samples consisting of less than ten hairs could be the subject of further study. Although the analysis of the HPV content of ten single hairs from just one individual at one time point could be considered of rather limited value, Table 2 clearly indicates that a substantial number of single hairs contained an identical set of HPV types to those found in the simultaneous hair sample composed of ten plucked hairs. Whether the absence or limited detection of HPV types in single hairs (Right 1 and 4 in Table 2) reflects true HPV negativity or should be attributed to sample heterogeneity is not known. Three types (HPV12, -14 and -19) were additionally found in the single hair samples compared with the combined hair samples during follow-up detection in this individual. This may be explained by the PCR effect of competition, which might play a greater role in the combined hair samples. In contrast, the additional finding of HPV15 in the regular hair sample could point to a non-homogeneous spread of the β-PV types over the eyebrow and suggests that the more hairs that are tested, the more β-PV types will be found. In our experience, the testing of ten eyebrow hairs is most convenient regarding the amount of labour for sample processing and the inconvenience for the test subjects.

The next step in the analysis of persistent β-PV infections is to investigate whether certain β-PV types or certain combinations of β-PV types are more likely to persist than others. The HPV type-specific data presented in Table 3 are too limited in number to draw any conclusions of this kind. Furthermore, it will be important to study whether, by analogy with mucosal HPV infections, individuals persistently infected with particular β-PVs, for instance those infected with the potentially high-risk β-PV types HPV5 and -8, are at increased risk of developing cutaneous SCC and precursor lesions such as actinic keratoses. The question remains, however, as to whether the association between cervical carcinogenesis and persistent mucosal HPV infection also applies to cutaneous SCC and persistent cutaneous HPV infections, especially with the high rate of persistent cutaneous infections that were found in this small cohort of immunocompetent individuals.

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