Lack of association between the presence and persistence of betapapillomavirus DNA in eyebrow hairs and betapapillomavirus L1 antibodies in serum

Elsemieke I. Plasmeijer,1 Rachel E. Neale,2 Peter O'Rourke,2 Kylie-Ann Mallitt,2 Maurits N. C. de Koning,3 Wim Quint,3 Petra G. Buettner,4 Michael Pawlita,5 Tim Waterboer,5 Adele C. Green2 and Mariet C. W. Feltkamp1

Correspondence
Elsemieke I. Plasmeijer
e.i.plasmeijer@lumc.nl

Received 19 January 2010
Accepted 28 April 2010

1Department of Medical Microbiology, Leiden University Medical Center, Leiden, PO Box 9600, 2300 RC Leiden, The Netherlands
2Cancer and Population Studies, QIMR, PO Royal Brisbane Hospital, Brisbane, QLD 4029, Australia
3DDL Diagnostic Laboratory, Fonteynenburghlaan 7, 2275 CX Voorburg, The Netherlands
4Skin Cancer Research Group within the North Queensland Centre for Cancer Research, School of Public Health, Tropical Medicine and Rehabilitation Sciences, James Cook University, Townsville, QLD, Australia
5Department of Genome Modifications and Carcinogenesis, German Cancer Research Center (DKFZ), Heidelberg, Germany

Betapapillomavirus (βPV) DNA and seroresponses are highly prevalent in the general population and both are frequently used as infection markers in epidemiological studies to elucidate an association with cutaneous squamous cell carcinoma (SCC). Little is known about the natural history of βPV infection and the aspects of infection that drive antibody responses. To investigate the relationship between these markers, this study assessed whether the presence or persistence of βPV DNA in eyebrow hairs and L1 antibodies of the same βPV type co-occurred more frequently than would be expected by chance in both a cross-sectional assessment and a longitudinal study. βPV DNA in plucked eyebrow hairs and L1 antibodies in serum were measured in 416 participants of the Australian community-based Nambour Skin Cancer Study in 1996. Similar data were available for a subset of 148 participants in 2003. Observed co-occurrence of βPV DNA and antibodies was compared with expected values based on prevalence. A case-wise concordance index was used to calculate the overall concordance of βPV DNA and antibodies of the same type. No significant associations were found between the presence or persistence of βPV DNA and antibody responses. The age and sex of the host did not influence the association, and nor did SCC status or a history of sunburns. It was concluded that βPV antibody responses in adults are not primarily driven by βPV infection as measured in eyebrow hairs. Other factors, such as viral load, may play a more pivotal role in the induction of detectable seroresponses.

INTRODUCTION

Human papillomaviruses of the genus *Betapapillomavirus* are non-enveloped, cutanotropic DNA viruses that may be associated with the development of cutaneous squamous cell carcinoma (SCC) (zur Hausen, 1999). So far, 31 different βPV types have been fully sequenced (De Villiers & Gunst, 2009; Pfister et al., 2003).

Epidemiological studies have shown that betapapillomavirus (βPV) DNA is frequently found in the hair bulbs of eyebrows and body hairs (Boxman et al., 1997), in normal skin swabs (Antonsson et al., 2000) and in biopsies from healthy people and transplant recipients without skin cancer (de Koning et al., 2009), as well as in SCC tumour tissue (Asgari et al., 2008; Plasmeijer et al., 2010; Rollison et al., 2008; Weissenborn et al., 2005). The presence of βPV DNA has been associated with the presence of SCC precursor lesions (actinic keratoses) and SCC (McBride et al., 2007; Plasmeijer et al., 2009; Struijk et al., 2003).
Antibodies against the βPV major capsid antigen L1 can be found in the serum of healthy controls as well as in patients with actinic keratoses and SCC, and have been associated with both tumour types in epidemiological studies (Bouwes Bavinck et al., 2000; Casabonne et al., 2007, 2009; Favre et al., 2000; Feltkamp et al., 2003; Karagas et al., 2006; Masini et al., 2003; Stark et al., 1998; Struijk et al., 2006; Waterboer et al., 2008, 2009).

Little is known about the association between βPV DNA in hair follicles and serum antibodies. It might be expected that antibodies arise as a result of infection of the hair follicles with βPV DNA, but the specific aspects of βPV infection that drive antibody responses are currently unknown. For example, the location, load and persistence of infection, as well as inflammation at the site of infection, may all be important in this respect (Favre et al., 2000; Weissenborn et al., 2005).

Only two studies so far have investigated βPV seroprevalence among people with known βPV DNA status. Struijk et al. (2006) reported a significantly higher prevalence of L1 seropositivity in those who were βPV DNA-positive in eyebrow hairs than in those in whom βPV DNA was not detected, whilst Andersson et al. (2008) reported that seropositivity was twice as common among people in whom DNA was detected for at least one βPV type in a healthy skin or SCC biopsy, and that 20% of people with βPV DNA were positive for L1 antibodies of the same type. In both studies, however, there was no statistical assessment of whether the associations found were higher than what would be expected on the basis of chance alone.

In comparison with βPVs, there is substantially more knowledge about alaphapapillomaviruses. For example, HPV-16 L1 capsid antibodies are known to be a valid measure of lifetime HPV-16 exposure, and the association between HPV-16 DNA in cervical biopsies and capsid protein antibodies is high (Dillner, 1999). Within a few months of the acquisition of viral DNA, a serological response is evoked in 50% of infected women. The majority of HPV DNA-positive women clear the infection within 12 months (Evander et al., 1995; Hildesheim et al., 1994; Ho et al., 1998). In women with a persistent presence of HPV DNA in samples taken on two different occasions, the percentage of seropositive women is higher than in women with HPV DNA diagnosed on a single occasion (Wideroff et al., 1995). Although the pathophysiology of βPV seems very different from that of the high-risk viruses with mucosal tropism, these data raise the possibility that βPV persistence might also be linked to a serological response.

To elucidate the role of βPV infection measured as the presence of viral DNA in the development of βPV L1 antibodies, we first aimed to assess whether detection of βPV DNA in eyebrow hairs was associated with seropositivity for the same virus types in a cross-sectional assessment. Secondly, we evaluated to what extent persistent DNA infection determined seropositivity. Due to a lack of information about long-term persistence of DNA, we measured βPV DNA persistence from 1996 to 2003 on the assumption that this is indicative of infection prior to antibody formation. The study was performed in the context of an Australian longitudinal skin cancer study.

**RESULTS**

**Population characteristics**

The mean age of the 416 participants with HPV DNA data and L1 antibody data from 1996 (the cross-sectional group) was 51 years and 50% were male. In total, 60 people (14%) had never had a painful sunburn, 184 people (44%) had experienced one to four painful sunburns in their life and 172 people (41%) had had five or more painful sunburns. Eighteen people (4%) were newly diagnosed with SCC between 1986 and 1996.

The mean age of the 148 people for whom HPV DNA data were available from both 1996 and 2003 (the longitudinal subgroup) was 50 years and 47% were male. Painful sunburns had the same distribution as in the whole cohort and four people (3%) in this group developed SCC between 1986 and 1996.

**Association between βPV DNA presence and L1 antibodies (cross-sectional group)**

The prevalence of βPV DNA in the cross-sectional group was 74%, with 53% of people being positive for more than one type (Table 1). There were 288 people (69%) with βPV antibodies and 51% had antibodies against more than one type. The prevalence of individual types ranged from 0 to 23% for DNA and 0 to 33% for antibodies (Table 1). Although the pooled case-wise concordance index between βPV DNA and L1 antibodies was significantly higher than DNA prevalence (P=0.30), for 12 of the 21 βPV types tested, the antibody prevalence was significantly higher than DNA prevalence (P<0.05), whilst for four of the 21 types, the DNA prevalence was significantly higher than for antibodies (Table 1). Although HPV-47 DNA was not detected in anyone in the cohort, 56 people (18%) had antibodies against HPV-47.

The pooled case-wise concordance index between βPV DNA and antibodies for the cross-sectional group was 0.18 (95% confidence interval (CI) 0.16–0.20). Examining individual βPV type concordance, we found all case-wise concordance indices to be ≤0.28 (see Supplementary Table S1, available in JGV Online). Of the 217 people with both βPV DNA and L1 antibodies, 114 people (53%) were positive for at least one βPV DNA and antibody of the same type, whereas 140 people (65%) were expected on the
basis of chance (P=0.15). Of the 308 people with at least one βPV DNA type, 114 people (37%) were positive for at least one of the corresponding βPV antibodies, compared with the 138 expected (P=0.045) (Table 2).

When we stratified by age (<50 years and ≥50 years), no significant differences were found in the proportion of people who were βPV DNA- and antibody-concordant for at least one type (P=0.62). In those aged <50 years, 42 people were concordant compared with the 52 expected (P=0.15), and, in those ≥50 years, 72 were observed whilst 86 were expected (P=0.14) (Table 2).

Stratification by SCC status did not show any significant differences in the proportion of people that were DNA–antibody concordant (P=0.70). In the group in whom SCC was detected, nine people were concordant compared with the ten expected (P=0.71), and, in the group without SCC, 106 were observed and 127 were expected (P=0.09). There was also no difference according to sex or number of lifetime sunburns (Table 2).

**Table 1.** Prevalence of βPV DNA in eyebrow hairs and βPV antibodies, overall and per βPV type

Values are numbers with percentages given in parentheses.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Cross-sectional study (n=416)</th>
<th>Longitudinal study (n=148)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall</td>
<td>308 (74)</td>
<td>288 (69)</td>
</tr>
<tr>
<td>HPV-5</td>
<td>46 (11)</td>
<td>36 (9)</td>
</tr>
<tr>
<td>HPV-8</td>
<td>44 (11)</td>
<td>137 (33)*</td>
</tr>
<tr>
<td>HPV-9</td>
<td>52 (13)</td>
<td>68 (16)</td>
</tr>
<tr>
<td>HPV-14</td>
<td>20 (5)</td>
<td>4 (1)†</td>
</tr>
<tr>
<td>HPV-15</td>
<td>73 (18)</td>
<td>102 (25)*</td>
</tr>
<tr>
<td>HPV-17</td>
<td>70 (17)</td>
<td>118 (28)*</td>
</tr>
<tr>
<td>HPV-20</td>
<td>42 (10)</td>
<td>47 (11)</td>
</tr>
<tr>
<td>HPV-21</td>
<td>10 (2)</td>
<td>86 (21)*</td>
</tr>
<tr>
<td>HPV-22</td>
<td>35 (8)</td>
<td>51 (12)</td>
</tr>
<tr>
<td>HPV-23</td>
<td>94 (23)</td>
<td>62 (15)†</td>
</tr>
<tr>
<td>HPV-24</td>
<td>69 (17)</td>
<td>68 (16)</td>
</tr>
<tr>
<td>HPV-36</td>
<td>73 (18)</td>
<td>53 (13)†</td>
</tr>
<tr>
<td>HPV-38</td>
<td>83 (20)</td>
<td>139 (33)*</td>
</tr>
<tr>
<td>HPV-47</td>
<td>0 (0)</td>
<td>56 (13)*</td>
</tr>
<tr>
<td>HPV-49</td>
<td>53 (13)</td>
<td>104 (25)*</td>
</tr>
<tr>
<td>HPV-75</td>
<td>11 (3)</td>
<td>64 (15)*</td>
</tr>
<tr>
<td>HPV-76</td>
<td>35 (8)</td>
<td>63 (15)*</td>
</tr>
<tr>
<td>HPV-80</td>
<td>42 (10)</td>
<td>78 (19)*</td>
</tr>
<tr>
<td>HPV-92</td>
<td>27 (6)</td>
<td>56 (13)*</td>
</tr>
<tr>
<td>HPV-93</td>
<td>70 (17)</td>
<td>13 (3)†</td>
</tr>
<tr>
<td>HPV-96</td>
<td>33 (8)</td>
<td>79 (19)*</td>
</tr>
</tbody>
</table>

*Significant difference in prevalence where antibody > DNA.
†Significant difference in prevalence where DNA < antibody.

**Association between βPV DNA persistence and L1 antibodies (longitudinal group)**

The prevalence of βPV DNA at baseline was 72%, with 48% of people being positive for more than one type (Table 1). Ninety-five people (64%) had βPV antibodies at baseline and 49% had antibodies against more than one type. The prevalence of antibodies for individual βPV types ranged from 0 to 18% for DNA and 0 to 33% for antibodies. Seventy-three people (49%) had persistent DNA detected for at least one βPV type in both 1996 and 2003 (Table 1).

The pooled concordance index describing the association between persistent DNA and antibodies was 0.13 (95% CI -0.005 to –0.27) and all individual case-wise concordance indices were ≤0.32 (Supplementary Table S2). Twenty-eight participants (38%) who had persistent βPV DNA also had L1 antibodies detected in 1996 for at least one βPV type, which was not significantly different from the number expected to occur by chance (n=25, P=0.61) (Table 2).
There were no significant differences in relation to age, sex, history of sunburns or SCC status among study participants (Table 2).

**DISCUSSION**

This study assessed the associations between both prevalence and persistence of βPV DNA and L1 antibodies, and found that neither DNA measure was predictive of antibody detection.

The overall βPV DNA prevalence of 74% we measured at baseline was lower than has been reported previously in an Australian population (91%; de Koning et al., 2009). βPV detection has been shown to increase with age (de Koning et al., 2009), so this difference is probably due to the younger age of our participants. The overall seroprevalence of 69% is higher than previously reported for Australia (Waterboer et al., 2009). As βPV seropositivity increases with age (Michael et al., 2008; Waterboer et al., 2009), the relatively high seroprevalence in our study was not due to age and remains unexplained.

We found antibodies to be very stable over 8 years, with only 16 people (11%) changing their overall βPV serology status between 1996 and 2003, and these people had mean fluorescence intensity (MFI) values very close to the cut-off value. We analysed our data using antibody status in 2003 and, due to the stability of these antibodies, found no difference in the results.

HPV-47 DNA was not found in the eyebrow hairs of any of the participants, but 13% were seropositive for HPV-47. Although sero-cross-reactivity, possibly with unknown βPV types, cannot be excluded, it is possible that the reference HPV-47 type represents a regional variant (Adachi et al., 1996). So far, HPV-47 DNA has only been found in an ongoing study in organ transplant patients using this method (M. N. C. de Koning, unpublished observation), and other βPV typing methods targeting different genomic regions of HPV-47 have also detected this type (Brink et al., 2005; Gheit et al., 2007). However, removal of HPV-47 from the analyses did not change the results of this study, because the proportions of DNA–antibody concordance as well as the case-wise concordance remained unaffected.

Neither the presence nor persistence of βPV in eyebrow hairs was associated with the detection of L1 antibodies. All type-specific and pooled concordance indices were low, and there were no consistent differences in the number of people expected to be concordant and the number observed. Stratification by age, sex, history of sunburns or SCC did not alter these findings.

Two other studies have addressed the relationship between βPV DNA and antibodies. One study found a significantly higher prevalence of L1 positivity in those who were βPV DNA-positive than in those in whom βPV DNA was not detected (Struijk et al., 2006). Of the 37 participants in that study with both βPV DNA and L1 antibodies, 32% were positive for the same type (Struijk et al., 2006). However, we would expect some people to be positive by chance alone and, without knowledge of this expected number, the results are difficult to interpret. We found that 53% of people who were DNA- and antibody-positive had at least one virus type where both measures were positive, but this was not higher than expected based on the prevalence of DNA and antibodies. A second study found that seropositivity was twice as common among people who had DNA...
detected for at least one \( \beta PV \) type in healthy skin or SCC on biopsy, and that 20% of people with \( \beta PV \) DNA were positive for L1 antibodies of the same type (Andersson et al., 2008), but again there was no reference to an expected value. Although we found that 37% of people with \( \beta PV \) DNA in eyebrow hairs were antibody-positive for at least one type detected, this was not higher than expected by chance.

There are several possible explanations for our observed lack of association between \( \beta PV \) DNA and L1 antibodies. It may be that the presence and/or persistence of \( \beta PV \) DNA we measured was not indicative of infection many years prior to 1996 and that the antibody response was provoked earlier in life, when the DNA was not present. However, a previous study showed that antibody responses against \( \beta PV \) types are rare in childhood and have their peak prevalence between 40 and 60 years for women and between 50 and 70 years for men, suggesting that antibodies arise at a time closer to our measure of infection (Michael et al., 2008). Secondly, it is possible that antibodies are detected for multiple types due to cross-reactivity, without there being infection of the skin with those types, and true type-specific seroresponses may be lower than measured. The high multiplicity of L1 antibodies and the significantly higher prevalence of antibody positivity than \( \beta PV \) DNA positivity for 12 types may support this hypothesis. Alternatively, \( \beta PV \) antibody responses may be associated with the \( \beta PV \) load rather than simply with the presence or absence of viral DNA. \( \beta PV \) loads, shown as the number of HPV copies per infected cell, are known to be much lower when compared with the alphapapillomavirus types (Weissenborn et al., 2005), but increases in load might be important in evoking an antibody response.

\( \beta PV \) DNA detection in eyebrow hairs has been used as a convenient marker of infection in epidemiological studies, but it is unclear to what extent this is indicative of pathologically relevant skin infection. Although we and others have shown some association between \( \beta PV \) DNA found in eyebrow hairs and in biopsies of cutaneous SCC and the perilesional skin (Asgari et al., 2008; Plasmeijer et al., 2010; Rollison et al., 2008), the prevalence in eyebrow hairs is substantially higher than in other tissues. Thus, it seems likely that a high proportion of infections in eyebrow hairs do not evoke an antibody response.

As the induction of an immune response often requires additional signals, inflammation accompanying \( \beta PV \) infections might be an important factor in evoking seroresponses. Favre et al. (2000) showed the induction of HPV-5 seroresponses upon second-degree burns of the skin, as well as in patients with autoimmune bullous diseases and psoriasis, all diseases with prominent inflammation (Favre et al., 2000). \( \beta PV \) infections as detected in eyebrow hairs are not known to be accompanied by inflammation. However, if eyebrow hairs are indicative of infection at other sites, we might expect that our association between \( \beta PV \) and antibody detection would be altered by a history of sunburns or SCC. We did not find an effect, however, of previous sunburns or SCC on the association between \( \beta PV \) DNA and L1 antibodies. Possibly, inflammation in the context of most SCC and actinic keratoses is small compared with those conditions examined by Favre et al. (2000).

In conclusion, we did not find a meaningful association between \( \beta PV \) DNA presence or persistence in eyebrow hairs and \( \beta PV \) L1 antibodies in serum. This lack of association highlights the need for a better understanding of the natural history of \( \beta PV \) infection and the ways in which infection induces an immune response before associations between measures of \( \beta PV \) infection and disease can be elucidated.

**METHODS**

**Study population and sample collection.** Participants were an unselected subset of the study population of the Nambour Skin Cancer Study described in detail previously (Green et al., 1996, 1999). Briefly, in 1986, 2095 of 3000 randomly selected residents of Nambour, a sub-tropical township in Australia (latitude 26° S), aged 20–69 years, participated in a skin cancer prevalence survey. From 1992 to 1996, 1621 of these participants took part in a trial of sunscreen application and \( \beta \)-carotene supplementation for the prevention of skin cancer. In 1996, 507 unselected participants took part in a sub-study aiming to understand the association between HPV and skin cancer (Boxman et al., 2001). Ten eyebrow hairs were plucked from each participant and blood was drawn where possible. For the present study, we used the data from 416 people for whom both \( \beta PV \) DNA and antibodies were available from 1996 (herein called the ‘cross-sectional group’). To analyse the association between persistent \( \beta PV \) DNA and seroreactivity, we used data from 148 people for whom \( \beta PV \) DNA and serum antibodies from 1996 and also from 2003 were available (herein called the ‘longitudinal group’). Skin cancer follow-up took place until 31 December 2007. Ethical approval for all aspects of the study was obtained through the Bancroft Centre Human Research Ethics Committee, Queensland Institute of Medical Research, Australia.

**DNA isolation, PCR and hybridization.** DNA from eyebrow hairs was isolated according to a method described previously (Boom et al., 1990). \( \beta PV \) detection and genotyping were performed using a reverse hybridization assay (RHA) as described by de Koning et al. (2006). All amplimers generated with the broad-spectrum PCR were analysed with an RHA that permitted specific detection and identification of 25 established \( \beta PV \) genotypes of which the following 21 were used: HPV-5, -8, -9, -14, -15, -17, -20, -21, -22, -23, -24, -36, -38, -47, -49, -75, -76, -80, -92, -93 and -96. The RHA was performed according to the manufacturer’s instructions [Skin (beta) HPV Prototype Research Assay; Diassay BV].

**Multiplex serology.** Serum samples were tested for the presence of antibodies to the major capsid antigen L1 for the 21 \( \beta PV \) types described above by multiplex serology. This is an antibody detection method based on a glutathione S-transferase capture ELISA, in combination with fluorescent bead technology (Andersson et al., 2008; Waterboer et al., 2005, 2009). Positive serology cut-off points were standardized at 200 MFI units.

**Statistical analyses.** We calculated the prevalence of \( \beta PV \) DNA and antibodies for any \( \beta PV \) type overall and for each of the 21 genotypes
tested for both DNA and antibodies. To test for significant differences between DNA and antibody prevalence of the same genotype, we used McNemar’s test. Persistent DNA was defined as having βPV DNA detected for the same type in 1996 and 2003, as we described previously (Plasmeijer et al., 2009).

To assess the associations between βPV DNA and antibody detection in the cross-sectional group, we calculated case-wise concordance, defined as the conditional probability that someone is positive for antibodies from a specific βPV type given that they are βPV DNA-positive for that same type. This was estimated as the ratio of the number of discordant people (positive for DNA and antibodies for the same βPV type) to the total number of discordant and concordant people (without βPV DNA and antibodies for the same type). It was interpreted similarly to a x² statistic. The standard error and 95% CI were estimated according to methods documented by Huang & Tai (2007). We estimated concordance separately for each βPV type and then calculated a pooled estimate across all types. To determine whether having persistent βPV DNA was associated with the presence of antibodies, we also calculated case-wise concordance between βPV DNA persistence and the presence of antibodies in both 1996 and 2003.

For each βPV type, we multiplied the proportion of participants positive for antibodies by the proportion positive for DNA to calculate the proportion that would be expected to be concordant for both measures purely by chance. To calculate the number of people who would be expected to be concordant for at least one type, these 21 proportions were summed and the product multiplied by the number of people measured. We compared the number observed with that expected, using a x² test to determine statistical significance. Because age, the number of sunburns and SCC status have all been shown to influence βPV seroreactivity (Favre et al., 2000; Feltkamp et al., 2003; Karagas et al., 2006; Waterboer et al., 2009), stratified analyses were performed for sex, age below and above the mean age, lifetime number of sunburns (0, 1–4, 5+) and SCC (detected between 1986 and 1996). The same approaches were used to determine associations between persistent DNA and antibodies detected in 1996 and 2003 (n=148). Because seroprevalence was very stable (overall, 89% of people remained either seropositive or seronegative) and analyses with 2003 antibodies showed the same results as with the 1996 antibodies, we have presented in this paper only the results of the 1996 βPV antibodies. Statistical analyses were performed with SAS version 9.1.

ACKNOWLEDGEMENTS

This study was supported by a grant from the Cancer Council Queensland. E. I. P. was supported by a travel grant from the Dutch Cancer Society (KWF). R. E. N. is supported by an NHMRC (Australia) Career Development Award. M. C. W. F. is supported by the Netherlands Organization for Health Research and Development (ZonMW, Clinical Fellowship grant 907-00-150). The authors state no conflict of interest.

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