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PSEUDOVIRION-BINDING AND NEUTRALIZING ANTIBODIES TO CUTANEOUS HUMAN PAPILLOMAVIRUSES CORRELATED TO PRESENCE OF HPV DNA IN SKIN

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SUMMARY

Whereas the antibody response to the anogenital Human Papillomaviruses (HPVs) is known to be mainly type-specific, correlated to presence of viral DNA and mainly directed to conformational epitopes of the virion, it is not known if this applies also to the antibody response to cutaneous HPVs. For 434 non-immunosuppressed patients with skin lesions (squamous cell carcinoma and basal cell carcinoma of the skin, actinic keratosis and benign skin lesions), we compared HPV DNA status with seroreactivity to HPV pseudovirions and to GST-L1 fusion proteins from HPV types 5, 6, 15, 16, 32 and 38. Biopsies from the skin lesions were tested for presence of HPV DNA using three different PCR methods, with typing by sequencing. Serum samples from subjects with HPV DNA positive biopsies and randomly selected serum samples from subjects with HPV DNA negative biopsies were also tested with neutralization assays with HPV5, 38 and 76 pseudovirions. Agreement of the three serological methods varied from poor to moderate. Type-specific seroprevalences among patients positive for the same type of HPV DNA (sensitivity of serology) was improved with the pseudovirion-based method (average of 40%, maximum 63%) compared to the GST-L1 method (average of 20%, maximum of 25%). Neutralization was the most sensitive assay for HPV38 (50%). In summary, also cutaneous HPVs appear to induce a type-specific antibody response that correlates with the presence of HPV DNA and that can be detected with improved sensitivity using pseudovirion-based serology.

INTRODUCTION

Human papillomaviruses (HPV) are the main causative factors for cervical cancer and several other human cancers (de Villiers *et al.*, 2004; zur Hausen, 2002). About 90% of the characterized HPV types belong to the *alpha, beta* and *gamma* genera. While HPV types in genus *alpha* mainly have a mucosal tropism, the viruses in the *beta* and *gamma* genera mostly have cutaneous tropism (de Villiers *et al.*, 2004). Cutaneous papillomaviruses are found both in healthy skin and in skin lesions such as actinic keratosis (AK) and squamous cell carcinoma of the skin (SCC) [Rewieved in (Feltkamp *et al.*, 2008)].

For anogenital HPVs, HPV serology is an important epidemiological tool to observe past and present HPV infections, as a substantial proportion of infected individuals develop type-specific serum antibodies to the major capsid protein L1 (Dillner, 1999). Seropositivity correlates past or present presence of the same type of viral DNA at the cervix (Dillner, 1999). Most of the antibody response is directed to conformational epitopes, with type-specificity being lost for denatured antigens (Dillner, 1999). Serology is also a tool to test for immunogenicity in HPV vaccination trials (Schiller & Lowy, 2009). The most widely used method in HPV serology is enzyme-linked immunosorbent assay (ELISA) measuring IgG antibodies to virus like particles (VLP)s (Kirnbauer *et al.*, 1994). VLPs spontaneously assemble after overexpression of L1 or L1 and L2 *in vitro* (Hagensee *et al.*, 1993; Kirnbauer *et al.*, 1992). HPV pseudovirions (PsV) mimic natural viruses in containing both the major capsid protein L1 and the minor capsid protein L2. PsV can also carry a genome and be infectious (Buck *et al.*, 2004). Neutralizing antibodies as detected in an HPV pseudovirion neutralization assay are protective against virus challenge, at least in case of mucosal HPV types (Pastrana *et al.*, 2004). For HPV16 and 18, the neutralization assay is known to be as

sensitive as ELISA, and also more specific, making it attractive for vaccine studies (Dessy *et al.*, 2008; Pastrana *et al.*, 2004).

The VLP ELISA and pseudovirion neutralization assay can only test for one HPV type at a time, resulting in that studies of HPV serology for multiple genotypes require large amounts of serum and labour. Therefore, immunoassays that measure HPV type-specific antibodies to several HPV genotypes simultaneously are becoming increasingly used. High-throughput multiplex HPV serology has used either VLPs (Dias *et al.*, 2005; Opalka *et al.*, 2003), capsomeres of L1 proteins expressed as glutathione S-transferase (GST) fusion proteins (Waterboer *et al.*, 2005) or pseudovirions bound to heparin-coated beads ((Faust *et al.*, 2010) as antigens. A competitive multiplex HPV serology method using HPV 6, 11, 16 and 18 L1 VLPs (Dias *et al.*, 2005; Opalka *et al.*, 2003) has been extensively used to measure antibody levels after vaccination with the quadrivalent HPV vaccine (Villa *et al.*, 2005). Lately, this competitive assay has been replaced with a method for measuring IgG binding directly to 9 HPV types as the competitive assay may underestimate the total protective antibody level (Opalka *et al.*, 2010). The use of the readily produced GST-L1 proteins as antigen enables testing for antibodies against a very large number of HPV types in a high-throughput multiplex system making it a widely used seroepidemiology tool (Michael *et al.*, 2008; Waterboer *et al.*, 2005). All conformational epitopes identified by monoclonal L1-VLP antibodies to mucosal HPV types are presented by GST-L1 proteins (Rizk *et al.*, 2008). However, studies evaluating the sensitivity to detect corresponding anti-HPV antibodies in patients positive for the DNA of cutaneous HPV types have reported a relatively low sensitivity (Andersson *et al.*, 2008; Plasmeijer *et al.*, 2010) and a lack of type-specific concordance between GST-L1 protein serology and presence of HPV DNA (Paaso *et al.*, 2011). Since there is no international standardization of the serological methods for cutaneous

HPV types, it is difficult to interpret and compare the results for different studies (Antonsson, 2012). Papillomavirus serology has been standardized only for HPV16 so far (Eklund *et al.*, 2012).

We have previously developed a high-throughput HPV serological method to detect IgG antibodies to mainly mucosal HPV types. The method is based on pseudovirions bound to heparin-coated Luminex beads and was validated by comparison with the HPV DNA status of the serum donors (Faust *et al.*, 2010). The aim of the present study was to expand the pseudovirion-based multiplex method to include more HPV types, in particular the cutaneous ones, and validate the method by comparison with presence of HPV DNA in the corresponding tumour tissue. As a clear correlation with presence of viral DNA has not been shown before for cutaneous HPVs, this aim also includes investigating whether cutaneous HPVs do indeed induce type-specific antibody responses. A second aim was to compare different methods by reanalyzing serum samples that had been analysed previously by the GST-L1 multiplex serology assays (Andersson *et al.*, 2008) with the newly developed Pseudovirion-based Luminex assay. As neutralization assays for cutaneous HPV types had also not been validated in relation to type-specific HPV DNA presence, we also wished to investigate whether a type-specific and neutralizing antibody response that correlates with presence of the same type of viral DNA could be demonstrated.

RESULTS

The design and production of pseudovirions was successful for HPV types 3, 15, 32, 33, 68 and 76. These did not differ in size or shape from previously described HPV pseudovirions (Faust *et al.*, 2010) (Figure 1). For unknown reason, HPV15 pseudovirions were not functional in the neutralization assay (data not shown), but were functional as VLPs.

In the Pseudovirion Luminex, the *beta* types had generally higher seroprevalences than the *alpha* types (on average 25% vs. 5% respectively). HPV types 5 (29%), 38 (26%) and 76 (27%) had the highest seroprevalence and HPV45 (0.7%) the lowest (Table 1). Among the mucosal types, HPV16 and 6 had the highest seroprevalences (both 6%). The HPV seroprevalences did not differen between the different patient groups (Table 1). The HPV type with highest seropositivity when tested with GST-L1 Luminex was HPV6 (32%), while only 6% of the subjects were seropositive for HPV6 in the Pseudovirion Luminex analysis (Table 1). Antibodies against HPV16 had similar prevalences with both methods (8% and 6% respectively). Antibodies against HPV5, 15, 38 and 32 were less prevalent with the GST-L1 method than with the Pseudovirion Luminex method (15% vs. 29%, 14% vs. 21%, 2% vs. 6% and 18% vs. 26% respectively) (Table 1).

The HPV seropositivity analyzed with all three methods was compared to the presence of HPV DNA in skin biopsies from the same donors (Table 2). Information about HPV DNA status was available for 427 subjects and the most commonly detected HPV were the *beta* types 5, 15, 38, and 76.

Thirty-eight percent of the patients who were positive for HPV5 DNA also had antibodies against HPV5 when using the Pseudovirion Luminex method, but only 15% when using the GST-L1 Luminex method and 30% when using the neutralization assay (Table 2). Thirty-nine

percent of the HPV38 DNA positive patients were also seropositive for HPV38 when using the Pseudovirion Luminex method, 25 % when using the GST-L1 Luminex method and 50% when using the neutralization assay. For HPV38, the neutralization assay was significantly associated with detection of HPV38 DNA (p=0.0002; Fisher´s exact test). HPV76 antibodies were detected by Pseudovirion Luminex in 63%, but by neutralization assay only in 13%, of the HPV76 DNA positive subjects. For HPV76, the pseudovirion-Luminex assay was significantly associated with detection of HPV76 DNA (p=0.03; Fisher´s exact test). GST-L1 serology data was not available for HPV76. The number of HPV15 seropositive patients out of the HPV15 DNA positive patients was low for both high-throughput serology methods (7% and 14% respectively) and the HPV15 neutralization assay was not functional (Table 2). Seroprevalences among subjects positive for some other HPV DNA type did not differ from the seroprevalences seen among HPV DNA-negative subjects (Table 2).

Comparison of the anti-HPV antibody response measured by the two high-throughput serology methods was possible for the HPV types 5, 6, 15, 16, 32 and 38 (Table 3a). Both continuous and categorical data obtained with the two different Luminex methods was compared by calculating R^2 and Kappa agreements. The continuous data agreement was best for HPV16 ($R^2 = 0.44$) and worst for HPV6 ($R^2 = 0.07$), with agreement for the other studied types having a correlation coefficient around $R^2 = 0.2$. The categorical data had an agreement that ranged from "poor" (HPV6, κ= -0.06), "slight" (HPV32, κ=0.17), "fair" (HPV15, κ= 0.38) to at best "moderate" (HPV38 κ=0.55, HPV16 κ=0.45 and HPV5 κ=0.45) (Landis & Koch, 1977). The Pseudovirion Luminex detected more positive samples compared to the GST-L1 method for HPV5 (74 vs. 12), HPV15 (56 vs. 23), HPV32 (22 vs. 4) and HPV38 (51 vs. 17) but fewer positives for HPV6 (23 vs. 135) and HPV16 (11 vs. 20) (Table 3a).

Patients DNA positive for HPV5, 38 and 76 (N= 40, 36 and 8 respectively) as well as 40

DNA negative patients for each type, were used to test the neutralization assay. Categorical

data obtained with the neutralization assay was compared with data from the Pseudovirion

and GST-L1 Luminex assays (Table 3b and c). The agreement between the high-throughput

Luminex methods and the neutralization assay varied from "fair" to "moderate". For the

Pseudovirion Luminex and the neutralization assay the Kappa value for HPV5 was 0.36 (fair)

and for HPV38 the Kappa value was 0.49 and for HPV76 0.41 (both "moderate") (Table 3b).

The GST-L1 Luminex and the neutralization assay agreed at a "moderate" level for HPV5, κ=

0.44 and "fair" for HPV38, κ= 0.24 (Table 3c).

DISCUSSION

In the present study, we have characterized the antibody response to cutaneous HPV types by expanding an already existing high-throughput method for HPV specific antibody detection (Faust *et al.*, 2010) by adding pseudovirions of three *beta* and three *alpha* HPV types to the assay and by developing pseudovirion-based neutralization assays. Our validation revealed that the pseudovirion-based antibody detection methods did correlate with presence of the same type of *beta* papillomavirus DNA, at least for HPV38 and 76. This suggests that the cutaneous HPVs induce a type-specific antibody response to conformational epitopes present on pseudovirions. Both the Pseudovirion-Luminex and GST-L1 assays were performed using multiplexing with fluorescent beads. The multiplexing enables simultaneous testing for a large number of viruses is of interest to enable more comprehensive serological studies. Theoretically, the fact that all the different antigens are incubated at the same time with the serum should also provide an increased ability to detect type-specific antibodies, as cross-reacting antibodies would absorb to the other antigens. However, the fact that the three serological methods evaluated in this study were not in good agreement, implies that cutaneous HPV serology is in need of further development and international standardization.

Although we tested 434 serum samples for antibodies against sixteen different HPV types, four of them belonging to genus *beta* (5, 15, 38, and 76) and twelve in the genus *alpha* (3, 6, 11, 16, 18, 31, 32, 33, 45, 52, 58 and 68), a limitation of the study is that only for 6 HPV types did we have data from both high throughput methods and that the neutralization assay was only performed for a subset of the study population (type specific HPV DNA positive cases and 40 controls per HPV type) as neutralization is very laborious. The comparison of neutralization assay with the two high-throughput Luminex serology assays is therefore limited.

In the present study population, antibodies to *beta* HPV types were more prevalent than antibodies to *alpha* types. This is expected, as the prevalence of cutaneous HPV types on the skin is usually greater than the prevalence of mucosal HPVs in the genital tract. Also, the age of the study group was high (mean age 72) and seroprevalences for cutaneous HPV types tend to continuously increase during lifetime, whereas antibodies against mucosal anogenital types are less common in elderly people (Michael *et al.*, 2008; Newall *et al.*, 2008).

We previously reported that then the GST-L1 Luminex results were compared to presence of HPV DNA in tissue samples (Andersson *et al.*, 2008), the sensitivity for HPV *beta* type serology was low. In this study, we found better agreement between HPV DNA in tissue samples and type specific antibodies with Pseudovirion Luminex than for GST-L1 for HPV5 and 38 (38% vs. 15% and 39% vs. 25% respectively). Neutralization should be a more specific method than measurement of binding IgG antibodies, and for HPV38 the neutralization assay was the most sensitive method as well. The difference in sensitivity to detect HPV76 between Pseudovirion Luminex and neutralization assay may indicate the existence of a non-neutralizing antibody response against this virus. For the pseudovirion-Luminex assay, a lack of association with presence of the same type of HPV DNA in the patient was only found for HPV type 15. This problem could be technical, but it is also possible that it may reflect a difference in biology. While different serotypes for the same genotype has not been described for anogenital HPV types, there is clear evidence that HPV 5 (a cutaneous HPV types) does contain at least 3 different serotypes corresponding to genetic subtypes of HPV5 (Favre *et al.*, 2000). If other cutaneous HPV genotypes also have several different genotypes, a lower concordance with presence of viral DNA would be expected.

When comparing agreements between methods using Kappa values, the highest agreement was "moderate". The largest discordance was for HPV6, where the antibody prevalence by Pseudovirion Luminex was almost 5 times lower than with the GST-L1 Luminex method and the agreement was non-existent. We have previously demonstrated validity of our Pseudovirion Luminex HPV6 test using serum samples from condylomata acuminata patients (Faust *et al.*, 2010) and found the method to detect HPV6 specific antibodies with high specificity, suggesting that the high HPV6 seroprevalences seen with GST-L1 Luminex may be due to low specificity.

The Pseudovirion Luminex serology method was found to, in general, have an improved sensitivity and specificity. We hope to expand the method to cover more HPV types by multiplexing and thus make it possible to perform high quality seroepidemiological studies for the cutaneous HPVs. The present study demonstrated that, in particular for the cutaneous HPVs, there are large differences in the results obtained with three HPV serology methods. International collaborative studies for validating and standardizing HPV serology also for cutaneous HPVs would thus be important to enable informative seroepidemiological studies for elucidating the natural history and possible role in human disease for the cutaneous HPV types.

MATERIALS AND METHODS

Patients and collection of data and samples

The enrollment of cases and controls is described in detail elsewhere (Andersson *et al.*, 2008). Briefly, the study included 434 immunocompetent patients attending dermatology clinics in Sweden (400 patients) or Austria (4 patients) (Forslund *et al.*, 2007). Cases were defined as having a histologically confirmed diagnosis of SCC (N=72, mean age 80 years, range 50-94), or BCC (N=160, mean age 73 years, range 34-93) whereas for the pre-malignant actinic keratosis, AK (N=81, mean age 75 years, range 53-95) clinical diagnosis was sufficient. The control patients had a variety of benign skin lesions (N=121, mean age 71 years, range 29-97), the most common being seborrhoeic keratosis (SK, N=62).

A serum sample was obtained from each subject and stored at -20°C. At the same visit as the serum sampling, two biopsies were collected from each patient, one 2 mm punch biopsy from the lesion and one from healthy skin 10-15 cm from the lesion. Before taking the biopsy, the skin was anesthetized and stripped with tape to avoid surface contaminations of virus (Forslund *et al.*, 2004). Seven individuals were excluded from HPV DNA testing (but were included in serology testing) because the skin had inadvertently not been stripped before taking of a biopsy (SCC, n=1; BCC, n=2; AK, n=1; Benign, n=3).

HPV DNA analysis

HPV DNA was analyzed as described (Andersson *et al.*, 2008). Briefly, the DNA from each punch biopsy was extracted using a phenol-free method (Forslund *et al.*, 1999). Sample adequacy was tested by PCR of the human β-globin gene (de Roda Husman *et al.*, 1995) (Forslund *et al.*, 2002). All samples were tested with four different HPV PCR methods in three different laboratories. All primers were located in the L1 open reading frame and typing

was done by sequencing of amplimers. If a sample was positive in at least one HPV test in at least one laboratory, the sample was scored as HPV DNA positive.

Pseudovirions

Expression vectors to produce HPV pseudovirion types 5, 6, 11, 16, 18, 31, 45, 52 and 58 were kindly provided by Drs. John Schiller and Christopher Buck, National Cancer Institute, Bethesda, Md, USA. Nucleotide sequences of those plasmids can be found at http://home.ccr.cancer.gov/lco/default.asp. Expression vectors to produce HPV pseudovirion types 3, 15, 32, 33, 68 and 76 (accession numbers in NCBI database x74462, x74468, NC_001586, M12732, DQ080079 and Y15174 respectively) were cloned using the same strategy as described for HPV38 (U31787) (Faust *et al.*, 2010). Animal PVs were not produced and used as negative control.

Pseudovirions (PsV) carrying secreted alkaline phosphotase (SEAP) reporter gene were generated by transfection of 293TT cells as described elsewhere (Buck *et al.*, 2004). Virus particles were adsorbed to carbon-coated grids, stained with uranyl formate and examined with transmission electron microscopy (TEM).

Pseudovirion Luminex

The method was performed as described (Faust *et al.*, 2010) in extended format that added pseudovirions of HPV types 3, 15, 32, 33, 68 and 76 to the previously established multiplex mix of pseudovirions of HPV 3, 5, 6, 11, 15, 16, 18, 31, 32, 33, 38, 45, 52, 58, 68 and 76). A Mean Fluorescence Intensity (MFI) of 250 was used as cut-off for seropositivity for all studied HPV types. All sera were tested in 2 serum dilutions (1:50 and 1:150) and were

scored as positive if they had >250 MFI in at least one dilution. The bead coupled with heparin only acted as a background control.

GST-L1 Luminex

The serum samples had been tested previously (Andersson *et al.*, 2008) for antibodies to the major capsid protein L1 of the HPV types 1, 5, 6, 8, 9, 10, 15, 16, 20, 24, 32, 36, 38 and 57, and to the early proteins E6 and E7 of the HPV types 8 and 38. Briefly, the GST-L1 Luminex serology used a glutathione S-transferase capture enzyme-linked immunosorbent assay in combination with fluorescent technology (Sehr *et al.*, 2001; Waterboer *et al.*, 2005; Waterboer *et al.*, 2006). For the six of these HPV types (HPV5, 6, 15, 16, 32 and 38) comparison with present Pseudovirion Luminex data was possible.

Neutralization assay

To detect HPV 5, 38 and 76 specific neutralizing antibodies in human serum, protocol by Pastrana *et* al (Pastrana *et al.*, 2004) was followed using a serum dilution of 1:50. Chemoluminescence was read for 0.2s per well using a Wallac Victor 1420 Multilabel counter. The serum was counted as neutralizing, if the secreted alkaline phosphatase signal was reduced by more than 50%.

Statistics

Agreement of categorical values between assays was quantified calculating Kappa values using GraphPad QuickCalcs online calculator. R^2 was calculated using Microsoft Excel to compare continues data of GST-L1 and PsV Luminex assays. Seroprevalences among subjects positive for the same HPV DNA type and HPV-negative subjects were compared using Fisher´s exact test (2-sided).

The study adheres to the Declaration of Helsinki Guidelines and was approved by the Ethics Committees of Karolinska Institutet and of Lund University, Sweden, and of Medical University of Vienna, Austria.

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FIGURES AND TABLES

Figure. 1. Transmission Electron Microscopy images of HPV pseudovirions. A- HPV 3, B- HPV 15, C- HPV 32, D- HPV 33, E- HPV 68, F- HPV 76.
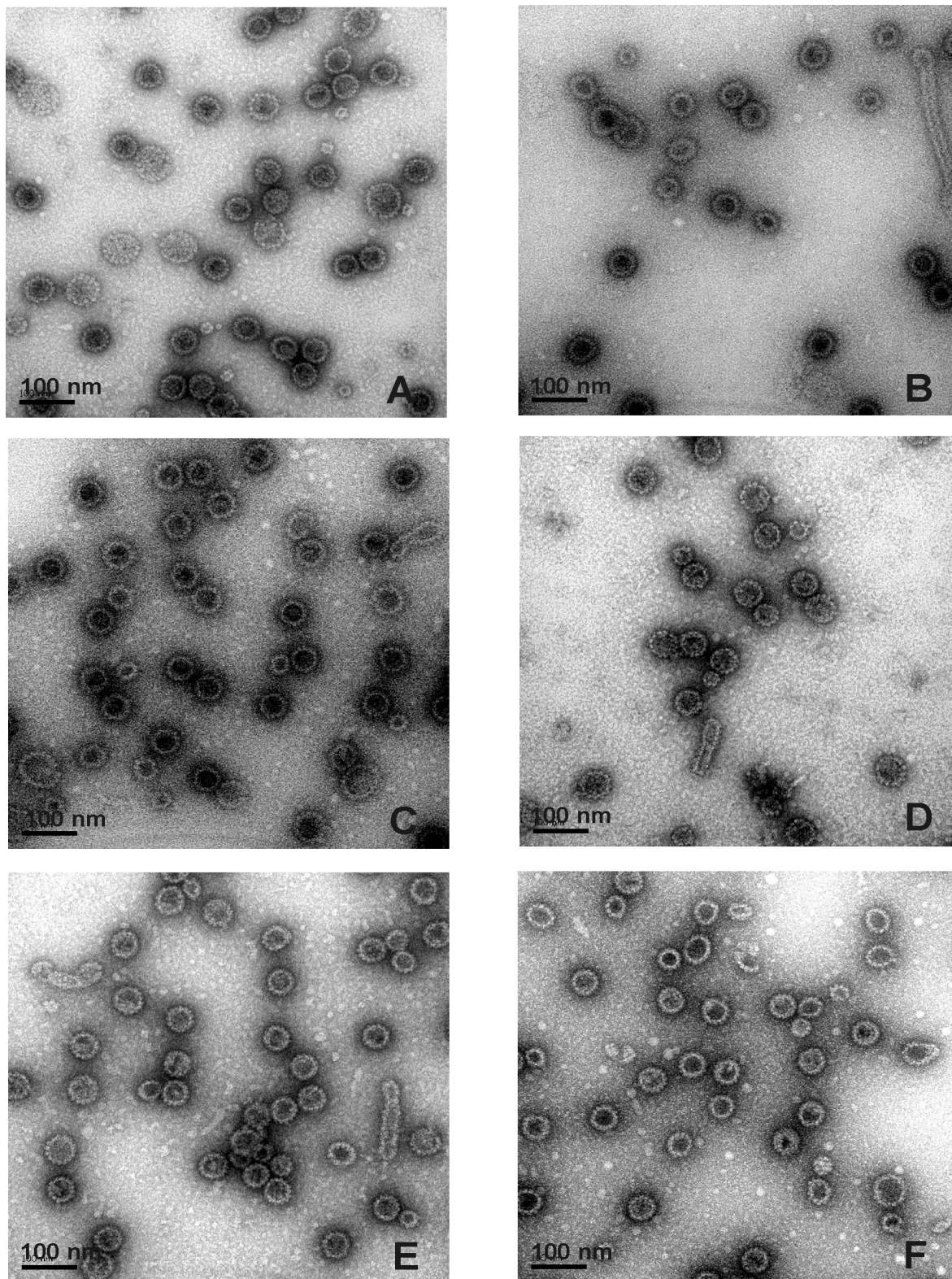
Table 1. Prevalence of antibodies against pseudovirions of 16 HPV types and 6 HPV types of GST-L1 proteins.

HPV type (genus)	Antigen	SCC N tot =72 N pos (%)	BCC N tot =160 N pos (%)	AK N tot = 81 N pos (%)	Benign Ntot=121 N pos (%)	Total Ntot=434 N pos (%)
HPV 3 (α)	PsV	3 (4)	5 (3)	2 (2.5)	6 (5)	16 (4)
HPV 5 (β)	PsV	19 (26)	49 (31)	20 (25)	40 (33)	128 (29)
	GST-L1	14 (19)	20 (13)	16 (20)	16 (13)	66 (15)
HPV 6 (α)	PsV	4 (5.5)	10 (6)	4 (5)	9 (7)	27 (6)
	GST-L1	19 (26)	53 (33)	25 (31)	42 (35)	139 (32)
HPV 11 (α)	PsV	2 (3)	5 (3)	0	3 (2.5)	10 (2)
HPV 15 (β)	PsV	17 (24)	29 (18)	22 (14)	25 (21)	93 (21)
	GST-L1	13 (18)	16 (19)	11 (14)	20 (17)	60 (14)
HPV 16 (α)	PsV	7 (10)	9 (6)	3 (4)	7 (6)	26 (6)
	GST-L1	8 (11)	11 (7)	4 (5)	12 (10)	35 (8)
HPV 18 (α)	PsV	1 (1)	2 (1)	2 (2.5)	3 (2.5)	8 (2)
HPV 31 (α)	PsV	4 (6)	5 (3)	5 (6)	3 (2.5)	17 (4)
HPV 32 (α)	PsV	0	9 (6)	6 (7)	10 (8)	25 (6)
	GST-L1	1 (1)	2 (1)	0	4 (3)	7 (2)
HPV 33 (α)	PsV	2 (3)	4 (2.5)	3 (4)	3 (2.5)	12 (3)
HPV 38 (β)	PsV	19 (26)	42 (26)	20 (25)	33 (27)	114 (26)
	GST-L1	18 (25)	25 (16)	15 (19)	22 (18)	80 (18)
HPV 45 (α)	PsV	0	1 (0.6)	1 (1)	1 (0.8)	3 (0.7)
HPV 52 (α)	PsV	1 (1)	5 (3)	3 (4)	9 (7)	18 (4)
HPV 58 (α)	PsV	3 (4)	5 (3)	5 (6)	8 (7)	21 (5)
HPV 68 (α)	PsV	1 (1)	10 (6)	4 (5)	4 (3)	19 (4)
HPV 76 (β)	PsV	21 (29)	41 (26)	23 (28)	32 (26)	117 (27)
Any HPV	PsV	45 (63)	98 (61)	41 (51)	81 (67)	265 (61)

Table 2. Type-specific comparison of HPV seropositivity analyzed by Pseudovirion and GST-L1 Luminex in patients with presence of HPV DNA in biopsies. Information about DNA status known for 427 subjects. Highest sensitivity in bold.

	Antibodies among patients DNA pos for the same HPV type (≥1 test)[1]			Antibodies among patients DNA pos for another HPV type (≥1 test)[2]		Antibodies among patients DNA negative for any HPV type (6 tests)[2]	
	Pseudovirion Luminex	GST-L1 Luminex	Neutralization assay	Pseudovirion Luminex	GST-L1 Luminex	Pseudovirion Luminex	GST-L1 Luminex
HPV5	**15/40 (38%)**	6/40 (15%)	12/40 (30%)	17/44 (27%)	5/44 (11%)	100/343 (29%)	55/343 (16%)
HPV15	1/14 (7%)	**2/14 (14%)**	NT	14/70 (20%)	9/70(13%)	77/343 (22%)	49/343 (14%)
HPV38	14/36 (39%)	9/36 (25%)	**18/36 (50%)**	10/48 (21%)	5/48 (10%)	90/343 (26%)	66/343 (19%)
HPV 76	**5/8 (63%)**	NT	1/8 (12.5%)	24/76 (32%)	NT	86/343 (25%)	NT

[1]No HPV 3, 6, 11, 18, 31, 32, 33, 52, 58 or 68 DNA was found. Only one patient had HPV16 in his biopsy but did not have antibodies to HPV16.
[2] From each individual two biopsies, one from lesion and one from healthy skin, were tested for HPV DNA in three laboratories. The individual was scored HPV DNA positive if at least one biopsy was positive in at least one of the laboratories. The individual was scored HPV-negative only if all biopsies were tested negative in all laboratories.
NT- Not tested

Table 3. Comparison of seroreactivity data obtained with different methods. Kappa value interpretation by Landis and colleagues (Landis & Koch, 1977).

A. Pseudovirion Luminex (PsV) and GST-L1 Luminex methods.

HPV type	R^2 of numerical data	Kappa agreement of categorical data	Psv + GST-L1 + N	Psv - GST-L1 – N	Psv + GST-L1 – N	Psv - GST-L1 + N	Total N
HPV5	0.22	0.45 Moderate	54	294	74	12	434
HPV6	0.07	-0.06 Poor	4	272	23	135	434
HPV15	0.19	0.38 Fair	37	318	56	23	434
HPV16	0.44	0.45 Moderate	15	388	11	20	434
HPV32	0.23	0.17 Slight	3	405	22	4	434
HPV38	0.23	0.55 Moderate	63	303	51	17	434

B. Pseudovirion Luminex (PsV) and Neutralization assay (Neutral.).

HPV type	Kappa agreement of categorical data	Psv + Neutral. + N	Psv - Neutral. – N	Psv + Neutral. – N	Psv - Neutral. + N	Total N
HPV5	0.36 Fair	13	45	6	16	80
HPV38	0.49 Moderate	14	46	9	7	76
HPV76	0.41 Moderate	7	30	5	6	48

C. GST-L1 Luminex and Neutralization assay (Neutral.).

HPV type	Kappa agreement of categorical data	GST-L1 + Neutral. + N	GST-L1 - Neutral. – N	GST-L1 + Neutral. – N	GST-L1 - Neutral. + N	Total N
HPV5	0.44 Moderate	8	58	11	3	80
HPV38	0.24 Fair	9	44	14	9	76