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**SERUM ANTIBODIES TO HUMAN PAPILLOMAVIRUS (HPV) PSEUDOVIRIONS
CORRELATE WITH NATURAL INFECTION FOR 13 GENITAL HPV TYPES**

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ABSTRACT

Background: Serology for human papillomaviruses (HPV) types -16 and -18 is established as an important tool for studies of HPV vaccinology and epidemiology. However, as there are a large number of oncogenic genital types of HPV there is a need for development of high-throughput, validated HPV serological assays that can be used for more comprehensive seroepidemiological studies and for research on multivalent HPV vaccines.

Objectives: To develop a multiplexed pseudovirion-based serological assay (PsV-Luminex) encompassing 21 HPV types and validate the method by correlating the serology with the presence of type specific HPV DNA in cervical samples.

Study design: Cervical swabs from 3,291 unvaccinated women attending organized cervical screening in Slovenia were tested with 3 different HPV DNA detection methods and presence of HPV DNA compared to presence of serum antibodies to pseudovirions from 15 genital HPV types (HPV-6,-11,-16,-18,-31,-33,-35,-39,-45,-52,-56,-58,-59,-68,-73).

Results: On average 51 % of the HPV DNA positive women were seropositive for the same HPV type that was detected in the cervical specimen. We found a strong correlation with presence of HPV DNA and antibodies to the same HPV type for 13/15 genital HPV types (median OR=5.7, CI 95%= 2.4-12.9). HPV-52 serology failed the validation and HPV-11 serology could not be validated because only a single woman was positive for HPV-11 DNA. The correlation between serology and HPV DNA status tended to be stronger among women infected with single HPV type (median OR=10.5, CI 95%=2.4-48.4) than among women with multiple HPV infections (median OR=4.6, CI 95%=1.8-11.7).

Conclusions: A multiplexed HPV PsV-Luminex assay has been developed and validated to correlate with natural HPV infection for 13 HPV types, thus enabling more comprehensive studies in HPV epidemiology and vaccine research.

1. Background

Persistent human papillomavirus (HPV) infection is established as a necessary cause for almost all cases of cervical cancer as well as for a proportion of vulvar, vaginal, penile, anal and oropharyngeal cancers (1, 2). Two highly effective vaccines are available against the two most oncogenic HPV types, HPV-16 and -18 (3, 4). However, there is still intensive research on HPV prophylactic vaccines as the exact mechanism of vaccine protection is not known (5) and as second-generation, multivalent HPV vaccines are under development (6).

Most genital HPV infections are transient and clear within 6-12 months (7). Therefore, HPV DNA testing cannot measure cumulative HPV exposure. Although not all HPV infections lead to seroconversion (8, 9), the antibody responses to the HPV capsid are known to be stable over time (also after clearance of HPV DNA) providing a useful measure of cumulative HPV exposure (10).

Most HPV serology studies performed to date have been restricted to HPV-16 and -18 (11), although some studies have also reported on seropositivity to other HPV types (12, 13). Multiplexed serology is a high-throughput method that could make it possible to study the seroepidemiology of multiple HPV types more routinely. So far, most multiplexed HPV serology studies have used GST-L1 fusion proteins as antigen (11). The type-specific antibody response to HPV is predominantly against conformational epitopes that are exposed on native virions, but not on disrupted virions. We have previously developed and validated a high-throughput, multiplexed HPV serology method based on mammalian cell-produced HPV pseudovirions (PsVs) for 10 HPV types (PsV-Luminex) (14). In the present study, we have expanded this method to include pseudovirions from 21 HPV types and 2 polyomaviruses (Merkel cell polyomavirus (MCPyV) and JC polyomavirus (JCPyV)). Validation of HPV serology for the less common HPV types is a challenge, as very large cohorts with both serum

sampling and reliable HPV DNA testing are required in order to obtain sufficiently large validation panels. We used a population of 3,291 women who had already been tested for presence of HPV DNA status for 15 genital HPV types (15, 16) to evaluate the performance of our extended multiplexed serology assay.

2. Objectives

To develop an expanded multiplexed HPV serological assay based on HPV pseudovirions and to evaluate the performance of the assay by comparing serology with HPV DNA status.

3. Study design

Study populations

In the present study, 3,321 women 20-64 years of age (average age 36 years) who participated in the National Cervical Cancer Screening Program (DP ZORA) in Slovenia were consecutively enrolled into the Slovenian HPV Prevalence Survey (SHPVPS) (15, 16). From each consenting participant, both a cervical smear and a blood sample were collected. Collection of cervical smears is described in detail elsewhere (16). Whole blood samples (5 mL) were stored at +4 °C for less than a week before centrifugation for 5 min at 3,000 rpm. The serum was aliquoted and stored at -30 °C until further analysis. Thirty women who had been vaccinated against HPV were excluded.

Two negative control serum panels were included: serum samples from 133 Swedish children not older than 12 years of age (average age 6 years) (17) and 71 serum samples from adult (average age 43 years) Swedish women who reported up to 1 life-time sexual partner (14, 18).

HPV DNA testing

HPV DNA testing and genotyping is described in detail elsewhere (15). Briefly, cervical samples were tested in parallel with Hybrid Capture 2 HPV DNA Test (hc2) (high-risk probe cocktail B) (Qiagen, Hilden, Germany) and RealTime High Risk HPV Test (RealTime; Abbott, Wiesbaden, Germany). All samples with concordant positive RealTime/hc2 results and all samples with discordant RealTime/hc2 results were further analyzed with the Linear Array HPV Genotyping Test (Linear Array; Roche Molecular Diagnostics, Branchburg, NJ), with an HPV-52 type-specific real-time PCR assay (INNO-LiPA HPV Genotyping *Extra* Test (Innogenetics, Ghent, Belgium)) and, if necessary an in-house GP5+/GP6+ PCR assay. In addition, 1,000 randomly selected samples were also tested for the presence of 37 HPV genotypes with Linear Array HPV Genotyping Test (Učakar et al., manuscript in preparation). Since the hc2 and RealTime assays do not include detection of HPV types -6, -11 and -73, the comparison of DNA prevalences and seroprevalences of these types were calculated only for samples that were tested with Linear Array HPV Genotyping Test and INNO-LiPA.

Serological HPV testing

Serology was performed as described (14). To minimise inter-assay variability, the antigens were produced in large batches that were bound to the beads, aliquoted and frozen until testing. We produced pseudovirions for 17 HPV types belonging to species alpha: 3, 6, 11, 16, 18, 31, 32, 33, 35, 39, 45, 52, 56, 58, 59, 68, 73 and for 4 HPV types belonging to species beta: 5, 15, 38, 76 as well as for MCV. Pseudovirions were generated by transfection of 293TT cells, as described (19). The sources of HPV and MCV PsVs expression constructs are listed in Table 1. JCV Virus Like Particles (VLPs) were a kind gift from Dr. K. Sasnauskas. Cut-off values to define seropositivity were calculated independently for each HPV type by analysing the mean fluorescence intensity unit (MFI) values obtained from 133 childrens' sera (≤ 12 years old). The cut-off algorithm recommended by the global HPV LabNet (20) (mean

MFI value of a negative control serum panel plus 3 standard deviations) was used. However, if the calculated cut-off value was less than 400 MFI, we used 400 MFI as cut-off. Children are commonly infected with polyomaviruses and for the polyomaviruses we used 400 MFI as cut-off. Seroconversions for cutaneous HPVs commonly occur when children start school and serum samples from pre-school age children are known to have low seroprevalences for cutaneous HPVs. The pseudovirions and the cut-off levels used are listed in Table 1. The average coefficient of variation was 22 % for the HPVs (range 17-25 %), 48 % for MCV and 103 % for JCV. The sensitivity of serology was defined as the proportion of subjects seropositive for a particular HPV type among the subjects testing positive for DNA of the same type.

Statistics

Odds ratios (OR) and 95 % confidence intervals (95 % CI) were estimated using the Statcalc module of Epi-info version 3.5.1 (shareware available at www.cdc.gov).

4. Results

In the control group of 133 children, the HPV antibody prevalence did not exceed 4% for any studied HPV type (Table 1). In the groups of adult women, HPV-16 had the highest seroprevalence (14.1 % in the control group of 71 women with ≤ 1 lifetime sexual partner and 25.2 % in the SHPVPS study population of 3,291 women). Among women with ≤ 1 lifetime sexual partner, seropositivity for more than one genital HPV type was uncommon (15.5 % positive for >1 type, 29.6 % positivity for a single type), whereas in the SHPVPS population seropositivity to multiple genital HPV types was more common (44.3 % positive for >1 genital type, 21.5 % positivity for a single type). Seropositivity for cutaneous HPV types was mostly found as seropositivity for only a single HPV type, in all studied populations.

HPV DNA status was available for 3,270 women from the SHPVPS study population. Among these, 515 were positive for at least 1 HPV type and out of these, 262 were positive for multiple HPV types. Among HPV DNA positive women, 51% (range 15-100 %) had antibodies to the same HPV type detected with PsV-Luminex method (Table 2). By comparison, among HPV DNA negative women 9% had antibodies to a particular HPV type and among women HPV DNA positive to some other genital HPV, 16% had antibodies to particular HPV type. The 9% seroprevalence among currently HPV-negative women does not necessarily constitute false positives, as women may have had previous HPV infections. The somewhat higher seroprevalence (16%) among subjects positive to another HPV type than the one tested for should not be interpreted as suggesting crossreactivity between HPV types, as the HPV types share the same mode of transmission. Previous studies validating HPV ELISA found that adjustment for sexual history explained most of the difference in seroprevalence between HPV-negatives and women positive for HPV DNA of another type (18). The median OR for association between HPV DNA and seropositivity to the same HPV type was 5.7 (CI 95 % 2.4-12.9) (Table 2). Only for HPV-52 was seropositivity not significantly associated with HPV DNA status (OR=1.8, CI 95 % 0.9-3.7). Only one woman was positive for HPV-11 DNA and evaluation of the HPV-11 serology was therefore not possible. The associations between DNA status and seropositivity tended to be stronger among women with a single HPV DNA infection (median OR=10.5, CI 95 % 2.4-48.4), compared to women with multiple HPV DNA infections (median OR= 4.6, CI 95 % 1.8-11.7). For HPV-59 the association was stronger for multiple infections and for HPV-16, there was no difference between single and multiple HPV infections.

Among the 253 women who had a cervical HPV DNA infection with a single HPV type, 175 were seropositive for at least one genital HPV type (Table 3). Of these, 81/175 (46%) had antibodies both to the HPV type that was present in the cervix and to at least one other genital HPV type. Seropositivity for the HPV type present in the cervix, but not to any other genital HPV type was found in only 14/175 (8 %) of these women. Seronegativity for all studied genital HPVs was present in 23/175 (13%) of these women and 57/175 (33 %) were positive for antibodies to another genital HPV type than the one present in the cervix.

5. Discussion

We have developed and validated a high-throughput serology method that contains the largest array of native HPV antigens (pseudovirions) so far described. Comprehensive HPV DNA testing of cervical swabs in a large number of women attending the cervical cancer screening program who participated in SHPVPS enabled us to evaluate the association of HPV type specific seroreactivity to the presence of cervical infection with the same HPV type, also for relatively uncommon HPV types.

Compared to our previous study (14), our method has a lower inter-assay coefficient of variation and an increased sensitivity. Since this study involved testing of several thousand serum samples and because activated Luminex beads are not recommended to be stored at +4 °C for more than 1 month, we had to change testing procedure. Rather than activating smaller amounts of beads many times, we chose to bind antigens sufficient for the entire study to the beads and freeze aliquots at -80 °C. The only antigen that appeared to be sensitive to freeze-thawing was JCV VLPs, where the coefficient of the variation was increased rather than decreased by the freezing. The improved sensitivity was largely attributable to the fact that cut-off values are calculated separately for every HPV type, using the international algorithm recommended by the WHO global HPV LabNet (20).

We found a low seroprevalence for genital HPVs among children, as expected (14, 17, 21, 22). The seroprevalence for MCV among children was around 30 %, which is also in agreement with other studies (23). Seroprevalences of JCV and MCV in the adult Slovenian women were also similar (around 65 %) to other studies of European adults (24).

The most commonly used method for HPV serology has been ELISA using HPV L1 VLPs, a method known to give comparable results to the PsV-Luminex method (14). Most ELISA studies have only used the HPV types included in the quadrivalent vaccine (HPV-6,-11,-16,-18) (11). Our HPV-16 and -18 seroprevalences are similar to those reported in a multi-country European study (25). The expected proportion of HPV DNA-positive women who have serum antibodies to the same type of HPV is around 50 % and this was also the case for most of the genital HPVs in the present study.

In our previous study, HPV-45 and HPV-52 PsV-Luminex did not perform adequately (14). In the present study, the HPV-52 test was still inadequate, but the HPV-45 test was now adequate. Possible reasons for the improvement include that the HPV DNA status was ascertained by multiple tests, which could have resulted in better validity or that a new batch of HPV-45 pseudovirions was used. Regarding HPV52, we found that the pseudovirions reacted well with type-specific monoclonal antibodies and that antibodies to these HPV-52 pseudovirions were clearly associated with cervical cancer in a large case-control study from Thailand (Faust et al, unpublished observation). HPV-52 is known to cross-react with several other probes in the Linear Array assay and in international proficiency studies of HPV genotyping, HPV-52 has been one of the most frequently misclassified types, in particular in relation to the closely related type HPV-58 (26). However, we have no indication of any sort of technical problem with the HPV-52 genotyping in this study and it is uncertain whether the problem with HPV-52 lies in the serology or the genotyping. At any rate, validation of each new batch of pseudovirions using positive and negative control serum panels from carefully genotyped women appears to be highly advisable.

We found a tendency for HPV seropositivity to associate more strongly with presence of the same type of HPV DNA, if there was only one type of HPV DNA present in the cervix. This tendency is of interest as it might suggest that in case of a natural single HPV infection, the immune system might respond more precisely compared to infection with multiple HPV types. This would agree with the fact that vaccination with multivalent HPV vaccines have been found to induce “cross-reactive” antibodies (27-29).

A limitation of our study was that the new HPV pseudovirions were not tested with neutralizing monoclonal antibodies to type-specific conformational epitopes to ensure that the pseudovirions adequately presented these epitopes. Another limitation was that we established HPV DNA status by testing cervical samples only resulting in that the assays for cutaneous HPV types could not be evaluated and that the HPV DNA status will have been underestimated, particularly for HPV-6 and -11, as these HPV types more frequently infect external genital sites than the cervix.

In conclusion, our expanded and validated high-throughput serology method using pseudovirions is likely to enable a more reliable use of comprehensive HPV serology in HPV vaccine research and studies of natural history and epidemiology of HPV.

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Competing interests: None declared

Ethical approval: The study has been conducted in accordance with the code of Ethics of the World Medical Association (Declaration of Helsinki) and has been approved by the Medical Ethics Committee of the Republic of Slovenia (Consent number:83/11/09) and was also approved by the Ethical Committee of Umeå, Sweden (Nr. 118/92, 95-2400 and 98/12).

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Tables and figures

Table 1. Seropositivity for HPV types and polyomaviruses in two control groups (Children \leq 12 years and women with \leq 1 lifetime sexual partner) and adult women participating in cervical screening.

Source of expression vectors	VIRUS	N=133 Children (\leq 12 years)	N= 71 Women (\leq 1 lifetime sexual partner)	N= 3291 Women attending cervical screening	Cut-off (MFI)
J. Schiller	HPV 6	2.3	9.9	19.2	591
J. Schiller	HPV 11	0.8	0.0	5.8	400
J. Schiller	HPV 16	2.3	14.1	25.2	400
J. Schiller	HPV 18	0.0	2.8	9.5	400
J. Schiller	HPV 31	0.0	1.4	17.3	400
J. Dillner	HPV 33	3.0	2.8	11.2	400
S. Beddows	HPV 35	2.3	12.7	12.5	400
S. Beddows	HPV 39	3.8	7.0	17.6	635
J. Schiller	HPV 45	0.8	1.4	5.7	400
J. Schiller	HPV 52	3.0	0.0	9.3	1400
R. Roden	HPV 56	0.8	4.2	10.4	657
J. Schiller	HPV 58	1.5	5.6	19.7	400
S. Beddows	HPV 59	0.8	7.0	12.5	400
J. Dillner	HPV 68	3.0	8.5	12.2	400
R. Roden	HPV 73	2.3	11.3	12.5	400
J. Dillner	HPV 3	0.0	2.8	5.0	400
Schiller	HPV 5	0.8	9.9	20.1	400
J. Dillner	HPV 15	0.8	11.3	13.1	400
J. Dillner	HPV 32	2.3	11.3	6.5	589
J. Dillner	HPV 38	1.5	1.4	9.3	400
J. Dillner	HPV 76	3.8	9.9	14.7	1066
K. Sasnauskas	JCV	12.0	49.3	65.1	400
C. Buck	MCV	31.6	49.3	68.2	400
	HPV any type	15.0	63.4	76.6	
	HPV single type	7.5	35.2	21.0	
	HPV any genital	11.3	45.1	65.8	
	HPV single genital	4.5	29.6	21.5	
	HPV any non genital	6.8	35.2	40.2	
	HPV single non gen.	6.0	26.8	23.1	
	HPV \geq 10 types	0.0	1.4	3.8	

Table 2. Associations between presence of cervical HPV DNA and HPV seropositivity for 15 genital HPV types among adult women participating in cervical screening.

HPV	Sero+/ DNA+ (N)	Assay sensitivity (%)	Sero+/ DNA- (N)	Sero+/ DNA- (%)	Sero+/DNA+ of other HPV (N)	Sero+/DNA+ of other HPV (%)	Probability that HPV DNA positive sample is seropositive for same type					
							ALL HPV DNA+		Single type DNA+		Multiple type DNA+	
							OR	CI 95%	OR	CI 95%	OR	CI 95%
6	7/11	63,6	257/1105	23.3	113/387	29.2	5,77	1,73-23,62	No single infection		5,77	1,73-23,62
11	1/1	100,0	67/1115	6	31/397	7.8	Only one woman DNA+		No single infection		Only one women DNA+	
16	68/120	56,7	752/3150	23.9	122/395	30.9	4,17	2,84-6,14	4,31	2,27-8,21	4,26	2,62-6,93
18	16/39	41,0	291/3231	9	68/476	14.3	7,03	3,5-14,03	17,05	5,03-60,29	4,73	1,87-11,62
31	47/83	56,6	517/3187	16.2	104/432	24.1	6,74	4,23-10,76	11,43	5,28-25,14	5,1	2,79-9,33
33	11/27	40,7	354/3243	10.9	63/488	12.9	5,61	2,42-12,89	10,54	2,45-46,83	4,06	1,35-11,71
35	8/9	88,9	399/3261	12.2	69/506	13.6	57,38	7,33-1226,48	No sero-/DNA+		43,08	5,17-952,08
39	19/40	47,5	557/3230	17.2	120/475	25.3	4,34	2,22-8,47	9,89	2,2-49,96	3,64	1,67-7,87
45	11/29	37,9	174/3241	5.4	38/486	7.8	10,77	4,68-24,45	22	5,88-83,97	7,06	2,17-21,57
52	10/65	15,4	295/3205	9.2	48/450	10.7	1,79	0,85-3,68	1,21	out of limits	2,06	0,88-4,66
56	8/22	36,4	332/3248	10.2	77/493	15.6	5,02	1,91-12,85	9,26	0-338,88	4,95	1,77-13,4
58	11/19	57,9	631/3251	19.4	130/496	26.2	5,71	2,12-15,59	10,72	1,85-79,83	4,32	1,23-15,13
59	17/41	41,5	393/3229	12.2	96/474	20.3	5,11	2,6-9,99	4,45	1,09-16,97	5,78	2,63-12,64
68	9/20	45,0	390/3250	12	88/495	17.8	6	2,28-15,68	12,37	3,66-43,66	1,27	out of limits
73	9/22	40,9	144/1094	13.2	60/376	16	4,57	1,77-11,66	Only 1 single infection		4,4	1,78-10,75
Average		51,33		9,00		16,00	9,29	2,89-99,74	10,29	2,97-75,37	7,18	2,13-84,72
Median		45,00		9,00		16,00	5,66	2,35-12,87	10,54	2,36-48,4	4,57	1,78-11,71

Table 3. Seropositivity of PsVs-Luminex for genital HPV types among adult women attending cervical screening, who had a cervical infection with a single HPV type.

HPV type	Single HPV DNA positive N	Seropositivity N (%)			
		Same HPV-/other genital HPV-	Same HPV+ /other genital HPV-	Same HPV+ /other genital HPV+	Same HPV-/other genital HPV+
16	44	9 (20)	2 (5)	23 (52)	10 (23)
18	13	2 (15)	1 (8)	7 (54)	3 (23)
31	34	3 (9)	4 (12)	19 (56)	8 (24)
33	9	0	1 (11)	4 (44)	4 (44)
35	2	0	0	2 (100)	0
39	9	2 (22)	1 (11)	5 (56)	1 (11)
45	11	0	0	6 (55)	5 (45)
52	19	4 (21)	1(5)	1(5)	13 (68)
56	2	0	1 (50)	0	1 (50)
58	7	0	3 (43)	2 (29)	2 (29)
59	11	2 (18)	0	4 (36)	5 (45)
68	13	1 (8)	0	8 (62)	4 (31)
73	1	0	0	0	1 (100)
Total	175	23 (13)	14 (8)	81 (46)	57 (33)