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Comparison of multiplex-serology and ELISA based methods in detecting HPV16 L1 antibody responses in paired saliva and serum samples of healthy men

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ABSTRACT

Human papilloma viruses (HPV) are a common cause of transient infections on mucosal surfaces, also in the oral cavity. Some infections remain persistent and can, especially with high risk HPV genotypes, lead to malignancies in the oral-oropharyngeal area. Our understanding of the natural course of oral HPV infections is limited, and the local host responses are poorly known. In this study we show that anti-HPV16L1 antibodies, the IgA response being most abundant, can be measured in saliva of asymptomatic males. HPV16L1 specific multiplex serology and commercial ELISA methods were compared and also the total salivary IgA levels measured. The total salivary IgA concentrations varied from 36 to 163 μg/ml. All the assays could detect anti-HPV16 IgA from saliva, but the correlation between assays varied from non-significant 0.22 to highly significant 0.81, $p < 0.01$. Salivary antibody responses did not correlate with the antibody responses detected in serum (Spearman correlations between −0.12 and 0.16) not even after adjusting the specific responses to differences in total IgA in saliva. Only six of 34 individuals were HPV16 DNA positive at the time of the sampling, but interestingly, three out of four with oral HPV16 DNA had salivary anti-HPV16 IgA responses below average. In conclusion, our results show that anti-HPV16 antibodies can be measured from saliva and the salivary response differs from that of serum. Individual differences in total salivary antibody concentrations may affect also the amount of HPV16 specific antibodies in saliva. Furthermore, different assay methods showed different specificities; thus comparisons between studies must be done with care.

1. Introduction

Human papillomaviruses are a common cause of transient infections on skin and mucosal surfaces. In some cases, however, the infection remains persistent, and these persistent infections, especially by the high risk HPV genotypes, may lead to malignant transformations of the epithelium. For example, almost 100% of cervix uteri cancers contain HPV DNA (Walboomers et al., 1999). Also oral HPV16 infections show similar fluctuating behavior as found in genital infections: most are asymptomatic and cleared, but some infections in some individuals remain persistent and can lead to malignancies, especially in the oropharyngeal area (Syrjanen, 2005).

HPV infection leads to production of antibodies against viral proteins. Serum antibodies against HPV capsid proteins are not thought to clear infections, but may provide protection against reinfection (Kirnbauer, 1996). Various methods (e.g. viral capsid protein based ELISA, luminex assays and viral neutralization assays) with different sensitivity and specificity (Kirnbauer, 1996) have been used to study the serology of HPV infection. In adults the serum anti-HPV capsid IgG is considered to be associated to lifetime cumulative exposure, while IgA is more related to recent or ongoing infection (Kirnbauer, 1996).

In saliva the main antibody type is secretory IgA (sIgA). sIgA is formed through the induction of common mucosal immune system, and thus differs from the specificity of antibodies in serum. The amount of IgG in saliva is low and it is mostly serum derived. Salivary antibodies, together with other salivary antimicrobial components, control many oral infections (Amerongen and Veerman, 2002; Lenander-Lumikari and Loimaranta, 2000). It is reasonable to assume that they may also

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modify individual susceptibility to, and progression of, oral HPV infections. After vaccination specific anti-HPV16 IgG antibodies can be detected in saliva (Handisurya et al., 2016; Pinto et al., 2016; Rowhani-Rahbar et al., 2009) but our knowledge of the salivary HPV16 antibodies in non-vaccinated individuals is very limited, and surprisingly few studies exist of sIgA, the most abundant antibody type in saliva (Passmore et al., 2007; Marais et al., 2001, 2006; Cameron et al., 2003).

The aim of this study was (1) to test different commercial ELISA platforms and multiplex serology in their ability to measure anti-HPV16 IgA and IgG antibodies in human serum and saliva and (2) to compare the salivary anti-HPV16 antibody levels to the total salivary antibody concentration as well as (3) to the HPV16 specific antibody levels in serum and (4) the oral and genital HPV16 DNA status of the individual.

2. Materials and methods

2.1. Study population

This study is part of the Turku University and Turku University Hospital Finnish Family HPV study, a prospective cohort study, assessing the dynamics of HPV infection in families (Rintala et al., 2005) that took place during years 1998–2008. Both parents participated in the study at the third semester of the pregnancy. The original study consisted more than 300 families whose oral and genital HPV prevalence was followed (FU) for six years. Our earlier studies have shown that in males oral HPV infection might affect HPV serology (Syrjanen et al., 2015); therefore saliva and serum samples from males were selected for this pilot study. Forty samples from 34 apparently healthy men (fathers-to-be) were selected based on the HPV16L1 seropositivity tested with Multiplex based method (Syrjanen et al., 2015; Waterboer et al., 2005). Their oral/genital HPV DNA status was not used as a selection criteria. HPV serology was scored positive when the antigen-specific Medium Fluorence Intensity (MFI) for HPV16 L1 value was 200 or higher or in a more stringent conditions 400 MFI or higher. Of the selected 40 samples, 9 had very high MFI values $>$ 1000, 10 had stringent MFI values ranging from 400 to 1000 and 21 had the MFI value in the range of 200–399 (i.e. the lower cut off value for HPV 16 seropositivity). In total 17 of the included men had HPV16 antibodies detectable in all their follow-up samples (Syrjanen et al., 2015). Saliva and serum samples taken at first or second visit during the FU were selected. Three additional serum and saliva samples collected at later FU visits (12, 24 and 36 months) from two men were included. This is because they had a significant increase in their HPV16L1 antibody (Multiplex) levels in serum during the FU and oral mucosal brush samples tested also HPV16 DNA positive at their 3-year FU visit. The oral and genital HPV DNA status at the time of sampling was known for 39/40 and 28/40 of the men, respectively (Kero et al., 2012). In total six samples were tested either genital or oral HPV16 DNA positive at the time of sampling. Other HPV types were identified in two of the HPV16 DNA positive samples and in 11 additional samples.

2.2. Sample collection

Serum and whole saliva samples were collected at the same visit, aliquoted and stored at −80 °C until used. The immunoglobulins are shown to remain stabile in deep frozen samples for decades (e.g. Gislefoss et al., 2009). A protease inhibitor aprotinin (Sigma-Aldrich) or cOmplete EDTA-free (Roche) was added into the saliva samples or dilution buffers, when samples were diluted. Before analysis, the saliva samples were thawed, centrifuged 5 min × 16,000*g*, and the supernatant used for analysis.

2.3. Anti-HPV antibody measurements

Three different methods were compared for anti-HPV16 detection, Multiplex based GST-fusion protein assay (Waterboer et al., 2005), a lateral flow assay Prevo-Check® (Abviris GmbH, Germany) and 3 commercial ELISA kits: Human anti-HPV16L1 IgG ELISA kit from Alpha Diagnostic International (Texas, USA, Kit A), Human papillomavirus type 16 L1-capsids (HPV16L1) antibody (IgG) ELISA Kit (Kit B), and Human anti HPV16 antibody (IgG) ELISA Kit (Kit C) both from Cusabio Biotech CO., Ltd. Kit A and Kit B were described to detect anti HPV16 L1 antibodies while Kit C was designed to analyze anti-HPV16 antibodies with no further specification of the applied antigen.

The antigen in the Multiplex assay was a pentameric GST-tagged recombinant HPV16L1 protein lacking ten N-terminal residues (Sehr et al., 2002). In the kit A, from Alpha Diagnostic International, the antigen was *E. coli* expressed His-tagged recombinant L1-protein (manufacturer's information). The antigens used in the ELISA assays B and C from Cusabio Biotech are not known. The antigen in lateral flow assay was HPV16 virus like particle (VLP) according to the information provided by the manufacturer.

The serum samples were analyzed with four different assay: the Multiplex assay, the lateral flow and the two ELISA assays detecting HPV16 L1 antibodies, Kit A and Kit B. Saliva samples were analyzed also with ELISA assay for total anti-HPV16 antibody (Kit C) but not with the lateral flow assay. For pairwise comparison of the assays serum samples were defined as positive or negative as instructed by the manufacturer (see Sections 2.3.1, 2.3.2, and 2.3.3) and Cohen's kappa values calculated.

2.3.1. Multiplex serology

Multiplex serology analysis for serum antibody responses against HPV16L1 was performed as described previously (Waterboer et al., 2005). Briefly, recombinantly expressed GST-tagged HPV16 L1 was affinity-purified onto glutathione-casein coupled fluorescent polystyrene beads (Luminex Corp., Austin, TX, USA). Serum (dilution 1:100) was incubated with antigen-loaded beads. Bound serum antibodies were detected by a biotinylated secondary anti-human IgG/IgA/IgM antibody (Dianova, Germany) as well as a reporter fluorescence Streptavidin-R-Phycoerythrin (Moss, Pasadena, MD, USA) and quantified by a Luminex 200 analyzer (Luminex Corp., Austin, TX, USA). The output was the median fluorescence intensity (MFI) of at least 100 beads per set measured. Detection of salivary anti-HPV16L1 antibodies was performed accordingly with the following adjustments: Saliva was applied in a 1:10 dilution and incubated with antigen-loaded beads at 4 °C overnight. A biotinylated anti-human IgA secondary antibody (Dianova, Germany) was used for specific detection of IgA. An MFI value of 200 was initially used as cutoff for seropositivity as described earlier (Syrjanen et al., 2015), but in this study also a higher, 400 MFI, cutoff values was tested. For salivary IgA no dichotomization was done but the raw data was compared.

2.3.2. Lateral flow

For serum samples a lateral flow assay designed to detect anti-HPV16 antibodies Prevo-Check® (Abviris Deutschland GmbH, Germany) was used. In the assay, 25 µl of serum was used and the test performed according to manufacturer's instructions. Briefly, the serum sample was mixed with a provided HPV-reagent. If serum contained anti-HPV16L1 antibodies they formed a complex with HPV-reagent and prevent the reaction between the reagent and detection antibodies in the test device. If the sample did not contain anti-HPV16L1 antibodies, HPV reagent bound to detection antibodies, which was seen as a colored band formation. The visual detection of presence or absence of colored band in test device defined positivity and negativity.

2.3.3. Anti-HPV16 ELISA assays

In the ELISA assays serum samples were diluted according to manufacturer's instructions and added into the pre-coated wells of microtiter plate. After incubation and washings, the bound antibodies were detected with HRP-conjugated anti human IgG and color formed from TMB-substrate. The positivity vs. negativity of the samples in

ELISA kits was defined according to manufacturer's instructions: Kit A contained Calibrators of 10, 5, 2.5 and 1 U/ml. The $OD_{sample}/OD_{calibrator1\ U/ml} > 1$ was defined as positive and < 1 as negative. Kits B and C had one positive and one negative control sample in addition to blank. The $OD_{sample}/OD_{negative\ control} > 2.1$ was defined as positive and < 2.1 as negative. Results were also plotted for visual evaluation of calculated cutoff values.

Different dilutions of saliva were tested and 1:10 and 1:3 dilution were selected for IgA and IgG, respectively. For saliva IgA analysis the detection antibody was changed to anti-human IgA–HRP conjugate (Dako, Copenhagen, Denmark, 1:5000 dilution in buffer provided in the kit). For salivary IgG measurements the controls included in the kits were used to define the cutoff values for positive and negative results according to manufacturer's instructions. In the salivary IgA measurements the provided controls did not respond to used anti-IgA-HRP conjugate and thus no dichotomization of the results was made.

2.3.4. Total IgA and IgG in saliva

The total IgA and IgG concentrations in saliva were analyzed with a capture ELISA assay (Lehtonen et al., 1984). Briefly, diluted saliva samples were added into wells in which the isotype-specific anti-human immunoglobulins were immobilized. After incubation and washing HRP-conjugated isotype specific antibodies were added, and binding detected with 1,2-phenylendiamine (Sigma Chemical Co.) as a substrate. Rabbit anti-IgA and -IgG and the corresponding antibodies conjugated with horseradish peroxidase were from Dako. Human control serum from NOR-Partigen (from Behringwerke AG, Marburg, Germany) was used as a standard.

2.4. Statistical analysis

Shapiro–Wilk test was used to check conformity of obtained results distribution to hypothetical normal distribution. The test showed that the data was not normally distributed ($p < 0.01$ to all data sets) and therefore non-parametric Spearman correlations were calculated. Differences between results obtained by ELISA kits and Multiplex assay were evaluated using the Wilcoxon Signed-Rank test because the data was non-normal. The statistical analyses were performed with IBM SPSS Statistics version 24.0.

3. Results

3.1. Saliva samples

All the ELISA kits and the Multiplex assay gave signals above blank with the saliva samples. All the measured results are shown in Fig. 1. The Multiplex results in salivary IgA measurement varied between 24 and 1346 MFI, and in the ELISA kits A, B and C the measured absorbance values varied from 0.21 to 2.4, 0.32 to 1.88 and 0.31 to 1.73, respectively. Clear differences (e.g. high response in one assay and low in other) were seen in individual sample level and there was statistically significant ($p \leq 0.001$, Wilcoxon Signed-Rank test) difference in IgA results obtained by different assay methods. Still, some statistically significant correlations were seen (Table 1). Surprisingly, the highest correlation (0.81) was found between the results of the kits B and C, measuring anti-HPV16L1 and total anti-HPV16 antibodies, and not between A and B which both claimed to measure anti HPV16L1 antibodies. Results measured by these two kits showed no significant correlation between each other (Table 1). The only statistically significant correlation of the Multiplex results (0.44, $p < 0.01$) was found between multiplex-assay and Kit B (Table 1). Both of these assays were designed to measure anti HPV16L1 antibodies. These results show that anti-HPV IgA can be detected in saliva, but selection of the assay method affects the outcome of the measurement.

To see if the obtained differences between ELISA assays are due to modifications that were needed to change the specificity from IgG to IgA, we tested ELISA kits also in saliva IgG measurement. For salivary IgG the raw data of the ELISA kits correlated between each other, and the best correlation (0.70, $p < 0.01$) was again between Kit B and C (Table 1). Significant positive correlations were found also between salivary anti-HPV16 IgG and IgA results (Table 1).

When calculated according to manufacturers' instructions (see Section 2.3.3) Kit A defined 15% (6/40) and Kits B and C 12.5% (5/40) of the saliva samples as anti-HPV IgG positive. Three of these positive samples were defined positive by all ELISA assays, three were positive only by Kit A, one by Kit B, one by Kit C and one by both B and C (Table 2). This suggests that the differences between ELISA assays may not be due to different sensitivities but more likely due to different specificities resulting from differences in coated antigen or other surface properties. Kappa-values show moderate to good repeatability. As in the IgA results, the best agreement (Cohen's kappa 0.77) was between Kit B and Kit C. The total agreement in salivary IgG results of all the ELISA assays was 85%, which is mainly due to large numbers of negative samples.

For salivary IgA any attempt to dichotomize the samples were considered to be arbitrary at this point. For comparison of the assays the samples were divided in tertiles according to their measured absorbance value (Table 3). Notably, in Multiplex assay all, and in ELISA assays five, of the six saliva samples from individuals who were defined HPV16 DNA positive at the time of sampling classified among the lowest or medium level antibody group (Table 3). Indeed, most (5/6) of the HPV16 positive individuals had salivary anti-HPV16 IgA values below the mean (not shown). The sample size is so small (six HPV16 DNA positive) and deviation so large that the significance of this difference remains to be verified in a larger cohort.

The amount of antibodies in saliva varies individually, for example, according to saliva flow rate. In general, slow flow rate results in higher concentration of antibodies. Therefore we measured the total salivary Ig concentrations that could be used to calculate the relative anti-HPV16 antibody amounts in the samples (Abs 450/ln total Ig or MFI/total Ig). The amount of salivary IgA varied between 36 and 163 μg/ml (median 87 μg/ml) and IgG between 3 and 170 μg/ml (median 11 μg/ml).

The relative HPV16/total IgA amount slightly changed the rank-order of the saliva samples (IgA, Table 3), but did not change the fact that samples from HPV16 DNA positive individuals were mostly grouped among the lower antibody groups. In ELISA results no changes were seen and in Multiplex assay one sample was grouped in to the highest tertile after adjustment (Table 3). This sample had relative low salivary IgA concentration, 45 μg/ml. In order to see whether there is a minimum amount of total IgA antibody in saliva that is needed for accurate HPV-specific results, correlation coefficients were calculated also without samples with lowest tertile of total sIgA (< 50 μg/ml). This did not improve the overall correlations between assays (not shown).

3.2. Serum samples

To compare measured antibody levels in saliva to the antibody levels in serum, HPV16 antibodies were measured from serum samples of the same individuals from the same time point by two of the ELISA kits, A and B, measuring anti-HPV16 L1 antibodies. An additional Lateral flow test, also designed to measure anti-HPV16 L1 antibodies, was included in the serum analysis. Samples were originally selected for the study by being seropositive in Multiplex assay (cutoff value 200 MFI; Syrjanen et al., 2015). In the ELISA absorbance values (A_{450}) from 0.44 to 1.11 (Kit A) and from 0.13 to 0.93 (Kit B) were measured (Fig. 1). Calculated cutoff values were 0.57 and 0.28, respectively. Visual examination of plotted results did not show any distinct populations to emerge, so cutoff values calculated as suggested by the manufacturers were used for further analysis.

Neither of the ELISA assays nor the lateral flow assay defined all serum samples as positive. The highest number of positive samples (17/

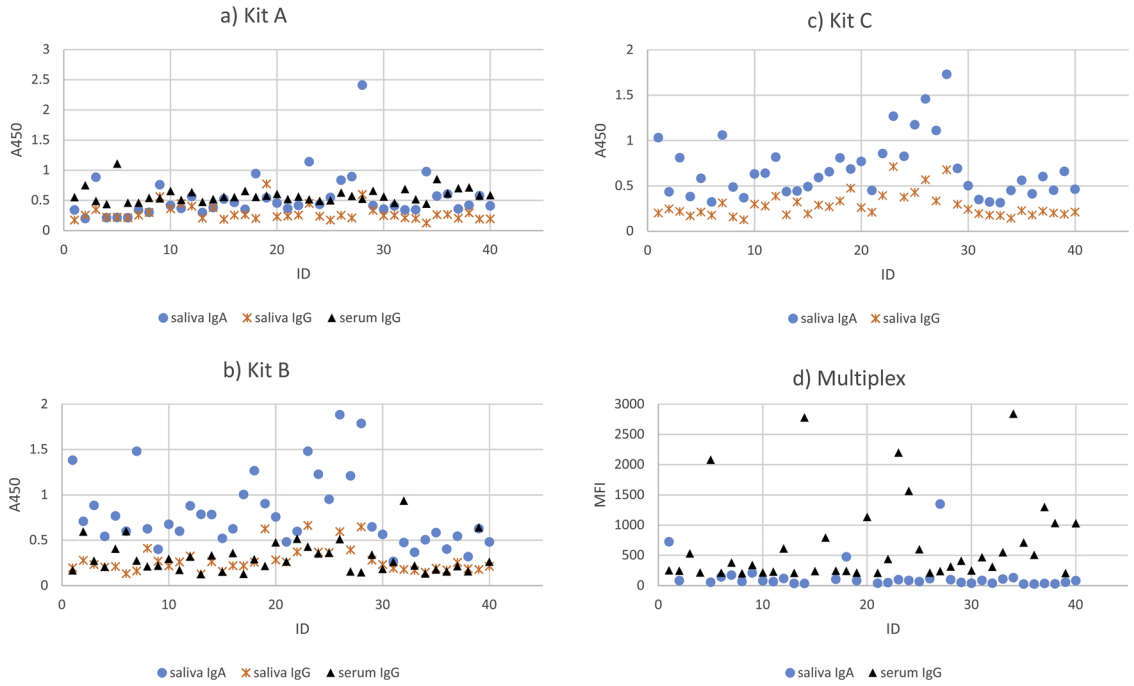

Fig. 1. Obtained serum and saliva anti-HPV IgA and IgG values by different ELISA kits (a–c) and Multiplex assay (d). MFI: mean fluorescent intensity, ID: id number of study subject.

Table 1
Spearman corerlation between results of saliva IgA and IgG anti-HPV16 antibody assays.

	Saliva IgA			Saliva IgG			
	Kit A	Kit B	Kit C	Multiplex	Kit A	Kit B	Kit C
Saliva IgA							
Kit A		0.23	0.48**	0.22	0.20	0.34*	0.40*
Kit B	0.23		0.81**	0.44**	0.17	0.51**	0.73**
Kit C	0.48**	0.81**		0.32	0.16	0.61**	0.80**
Multiplex	0.22	0.44**	0.32		−0.12	0.17	0.15
Saliva IgG							
Kit A	0.20	0.17	0.16	−0.12		0.48*	0.39*
Kit B	0.34*	0.51**	0.61**	0.17	0.48*		0.70**
Kit C	0.40*	0.73**	0.80**	0.15	0.39*	0.70**	

* $p < 0.05$.
** $p < 0.01$.

Table 2
Pairwise comparison and Cohen's kappa values of the salivary HPV16 IgG results measured by different assays.

		Kit A			Kit B		
		Positive	Negative	Cohen's kappa	Positive	Negative	Cohen's kappa
Kit B	Positive	3	2				
	Negative	3	32	**0.47***			
Kit C	Positive	3	2		4	1	
	Negative	3	32	**0.47***	1	34	**0.77****

* Moderate agreement, **Good agreement.

40) was found by ELISA Kit B and lowest with the lateral flow assay (6/40; Fig. 2). The two individuals who had genital HPV16 DNA at the time of sampling were seronegative by both ELISA assays and the lateral flow assay. Of the four individuals who were oral HPV16 DNA positive at the time of sampling three were defined as seropositive by both ELISA assays and one by lateral flow.

To test if the difference between Multiplex-serology and the other assays was only due to higher sensitivity of the Multiplex-serology assay, a higher cutoff value, 400 MFI, was used in the Multiplex assay. Then 19/40 (47.5%, Fig. 2) of the samples were positive. Neither of the individuals with the genital HPV16 DNA but three out of four individuals with oral HPV16 were defined seropositive also with this cutoff value.

For pair wise comparison of the serum IgG assays Cohen's kappa-values were calculated (Table 4) and they show relatively low repeatability between the assays (all below 0.4, Table 4). All the six samples that were positive in the lateral flow assay were positive also in multiplex serology, but only few of the positive samples in multiplex serology were positive in the lateral flow (Table 4). The samples that gave five highest MFI values in multiplex serology were among the lateral flow positive samples. It appears that the main difference between these assays could be the lesser sensitivity of the lateral flow assay. For other comparisons no such phenomena could be seen, suggesting that differences are not due to different sensitivity of the assays, but may relate to e.g. different epitopes in used antigens.

3.3. Comparison of saliva and serum antibody levels

Of the individuals whose serum samples were ELISA-positive less than half were defined as positive in saliva IgG tests, even after being adjusted to total IgG in saliva. When raw data were compared, no positive correlation was found between the salivary IgA or IgG and serum IgG values even when compared within one assay (Table 5). Instead, a weak negative correlation between Multiplex salivary IgA and serum IgG measured by Kit A was seen. To adjust to the individual differences in antibody concentrations in saliva, also the relative antibody concentrations (measured absorbance values/ln total IgG or IgA) were compared with serum IgG values but no correlation was found in this comparison (not shown).

3.4. Antibody kinetics of two individuals

Additional FU sera and saliva samples (12, 24, 36 month) from the two men testing oral HPV16 DNA positive at 36-month visit and having

Table 3
Comparison of rank order of saliva samples according to the HPV-specific and relative IgA responce in the saliva sample (IgA/ln tot IgA). Highest third = yellow, medium = rosa, lowest third = green.

IgA	IgA/ ln tot IgA	IgA	IgA/ln tot IgA	IgA	IgA/ln tot IgA	IgA	IgA/tot IgA	HPV status*			
ELISA KitA		ELISA Kit B		ELISA Kit C		Multiplex		Mouth	HPV type	Semen/ureter	HPV type
								HPV+	HPV16	ND	
								HPV+	HPV16	HPV-	
								HPV+	HPV16	ND	
								HPV+	HPV11&16	HPV-	
								HPV-		HPV+	HPV16
								HPV-		HPV+	HPV6,16,33
								HPV+	HPV18&59	ND	
								HPV+	HPV11	HPV+	HPV18
								HPV+	HPV82	HPV-	
								HPV+	HPV59	ND	
								HPV+	HPV43	HPV-	
								HPV-		HPV+	HPV6
								HPV-		HPV+	HPV6
								HPV-		HPV+	HPV6
								HPV-		HPV+	HPV51
								HPV-		HPV+	HPV43
								HPV-		HPV+	HPV33,56
								HPV-		HPV-	
								HPV-		HPV-	
								HPV-		ND	
								HPV-		HPV-	
								HPV-		HPV-	
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								HPV-		ND	
								HPV-		ND	
								HPV-		HPV-	
								HPV-		ND	
								HPV-		HPV-	
								ND		ND	

a significant increase in their HPV16L1 antibody levels (as measured earlier by Multiplex-serology; Syrjanen et al., 2015) were included in the study. The analysis of the serum samples revealed interesting difference between the ELISA assays used. The serum samples from ID1 were found negative by ELISA kit A but positive by ELISA kit B while the opposite was true for the serum samples from ID2. Also the lateral flow assay defined ID1 as positive and 2 as negative. All the samples were positive in Multiplex-serology even if the higher cutoff value

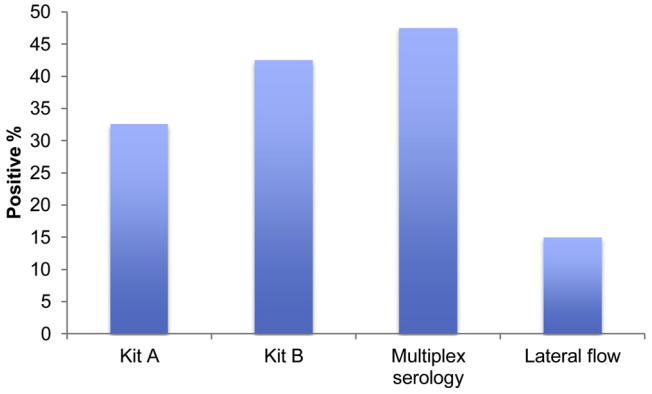

Fig. 2. The percentage of positive serum samples defined by ELISA Kit A, Kit B, Multiplex serology and lateral flow assays.

400MFI was used (Table 6).

In the saliva analysis highest absorbance results were measured for ID1 sample by Kit A while the Kit B again gave clearly higher IgA signals from ID1 samples than from the samples of ID2. This was true also to Multiplex assay, even though the Multiplex signals remained relative low (Table 6). Of the saliva samples the 12-month sample from ID1 was the highest in all assays, even though oral HPV16 DNA was present at the 36-month visit. This was true also for salivary HPV16 IgG measurements. This sample contained exceptionally high total IgG concentration (170 μg/ml) that can partially explain the positive result. Otherwise the total Ig amounts were comparable between the samples and do not explain the obtained differences. Instead it appears that the different assay methods may measure antibodies with different specificities and that different epitopes may be recognized by saliva and serum antibodies.

4. Discussion

In this study we showed that specific HPV16L1 antibodies can be detected in saliva by ELISA and Multiplex assays. Results from tested ELISA kits showed some degree of correlation between the assays but less correlation with Multiplex assay.

Salivary IgA and IgG results showed strong correlation indicating a possible non-specificity in the detection, especially in the results of Kit B and C. The anti-human IgA antibody used in the ELISA assays is, according to the manufacturer, tested not to have a cross-reactivity with the human IgG. The anti-human IgG used in Kit A was, according to the manufacturer, tested not to react with human IgA, but for Kit B and C no such information was available. Thus there is a possibility for cross-reaction with IgA in IgG reaction of Kit B and C.

Discordances between ELISA assays were seen in HPV16 serum analysis, as has been shown also before (Safaeian et al., 2012; Du et al., 2012). Despite the fact that the Multiplex and two of tested ELISA assays were designed to detect antibodies specifically against HPV16L1 antigens, they appeared to present different epitopes. This was suggested by follow up samples of two individuals where the tested assays clearly differed in recognizing antibodies of these individuals. Altogether this stresses the fact that comparison of results between studies performed with different assays must be done with care.

Poor correlation was found between HPV16 antibody activities in paired saliva and serum samples, even when measured by the same assay. In an earlier study a modest correlation was reported when HPV16 IgG antibodies in naturally infected subjects were compared to paired saliva and serum samples in HIV positive individuals (Cameron et al., 2003). The difference can relate to differences in study population, healthy vs. HIV-infected, since HIV infection is known to modulate the mucosal immune system (Heron and Elahi, 2017). Poor to modest correlation between oral and serum HPV16 IgG antibodies is reported also in studies where oral fluid, enriched with gingival fluid containing serum filtrated IgG, was used (Marais et al., 2001). Clear correlation between saliva and serum HPV16 IgG antibodies are reported after vaccination when highly immunogenic antigens induce strong responses (Handisurya et al., 2016; Pinto et al., 2016; Rowhani-Rahbar et al., 2009), but this appears not to be the case in natural infections.

Total salivary Ig amounts differ significantly also between individuals, as was seen in our study. Several factors, the flow rate being most obvious, but also time of sampling, eating/drinking before sampling, stress, cigarette smoking etc. affect the sIgA concentration in saliva (Brandtzaeg, 2013). In our study the difference between the lowest and highest IgA concentration was more than 4.5 times. There is a strong negative correlation between sIgA concentration and saliva flow rate (Brandtzaeg, 2013; Prodan et al., 2015), which can lead to wrong negative results if the saliva flow rate is high, and therefore relative concentrations (specific IgA/tot IgA) are often used. The pitfall is that high total IgA concentrations can diminish the calculated specific

Table 5
Spearman correlation between results of saliva and serum anti-HPV16 antibody assays.

	Serum IgG		
	Kit A	Kit B	Multiplex
Saliva IgA			
Kit A	−0.01	0.08	0.22
Kit B	−0.04	0.16	−0.08
Kit C	0.07	0.11	0.06
Multiplex	−0.36*	0.02	−0.12
Saliva IgG			
Kit A	0.12	−0.01	0.01
Kit B	0.14	0.23	0.01
Kit C	0.15	0.27	0.14
Serum IgG			
Kit A		0.13	0.08
Kit B	0.13		0.10
Multiplex	0.08	0.10	

* $p < 0.05$.

Table 4
Pairwise comparison and Cohen's kappa values of the serum HPV16 IgG results measured by different assays.

		Kit A			Kit B			Multiplex		
		Positive	Negative	Cohen's kappa	Positive	Negative	Cohen's kappa	Positive	Negative	Cohen's kappa
Kit B	Positive	8	9							
	Negative	5	18	**0.26***						
Multiplex	Positive	8	10		10	8				
	Negative	5	17	**0.22***	7	15	**0.24***			
Lateral flow	Positive	1	5		5	1		6	0	
	Negative	12	22	**−0.13**	12	22	**0.27***	12	22	**0.35***

* Fair agreement.

Table 6
The HPV16 antibody results of serum and saliva samples of two individuals from different time points and the total amount of antibodies in saliva samples. Results above the cutoff value of the assay are highlighted in yellow.

	Serum				Saliva									
					IgA			total IgA	IgG			total IgG	HPV DNA#	
ID/months	Kit A	Kit B	Lateral flow	Multiplex*	Kit A	Kit B	Multiplex	µg/ml	Kit A	Kit B	Kit C	µg/ml	mouth	semen/ureter
1/pre	-	+	-	+	0,42	0,60	47	87	0,26	0,37	0,39	42	HPV -	HPV -
1/12	-	+	+	+	1,14	1,48	96	147	0,45	0,66	0,71	170	HPV -	ND
1/24	-	+	+	+	0,44	1,23	83	75	0,24	0,37	0,38	17	HPV 18 & 59	ND
1/36	-	+	+	+	0,55	0,95	64	65	0,18	0,36	0,43	14	HPV 16	ND
2/pre	+	-	-	+	0,57	0,58	24	108	0,26	0,19	0,23	13	HPV -	HPV -
2/12	+	-	-	+	0,61	0,40	25	147	0,27	0,17	0,18	29	HPV -	ND
2/24	+	-	-	+	0,36	0,54	34	120	0,21	0,26	0,22	16	HPV -	ND
2/36	+	-	-	+	0,42	0,32	26	163	0,29	0,18	0,20	35	HPV 16	ND

HPV16 response that is biologically relevant, or vice versa. Calculation of relative HPV16 IgA values in our study changed the rank order of saliva sample IgA amounts and, for example, the sample from a genital HPV16 DNA positive individual showed high relative but low total HPV16 IgA response. Three of four oral HPV16 DNA positive samples were rather low both in absolute and relative IgA activities. Thus no link could be shown with salivary IgA activation and the presence of oral HPV16 DNA with this small number of samples. Instead it would be intriguing to speculate that high amount of oral anti-HPV16 IgA may protect against new infections.

In the healthy mouth the IgG concentration is very low, and therefore the specific IgG responses may be below detection level despite the high amount of specific antibodies in the serum. Periodontal disease or mucosal damage are associated with elevated salivary IgG concentrations (Brandtzaeg, 2013), but also persistent oral HPV infection is reported to be associated with elevated salivary IgG concentration (Haukioja et al., 2014). In the present study the IgG concentrations varied from 3 to 170 µg/ml, but the number of HPV16 DNA positive samples was so low that such association could not be detected. More than 50-fold differences in IgG concentration affects the specific antibody assay results as was demonstrated in our study. The saliva sample with the exceptional high total IgG amount was the only positive sample in all used assay. Thus, in addition to specific Ig activity other parameters need to be measured from saliva samples to increase the comparability of obtained results, and still the normalization of samples remains difficult.

Measuring HPV16 positivity of saliva samples is complicated also by the fact that a negative control population is difficult to find and define, because most individuals get HPV infection at some point of their life, and being DNA-negative at one time point does not exclude earlier infection, and thereby induced antibodies. Thus, dichotomizing results is challenging. The IgA antibodies are thought to be shorter lived that IgG, which can persist for years (Wang et al., 2000). Thus, in theory in follow up studies individuals repeatedly HPV DNA-negative could be identified and pooled saliva from such individuals may function as a negative control. In this study we used negative controls designed for serum samples in saliva IgG assays. For IgA different artificial cutoff values were tested, but no conclusive cutoff values were defined. Some assays studying the oral fluid antibodies have used pooled samples from young children as negative control (Marais et al., 2006) but also in such population individuals with high reactivity for HPV16 antigens are reported (Cameron et al., 2003). In addition, the salivary Ig concentrations differ between adults and young children (Brandtzaeg, 2013), and thus children are not optimal controls for adult saliva assays.

Subjects for our study were selected based on being HPV16L1 seropositive by Multiplex-serology in a previous study (Syrjanen et al., 2015). The ELISA assays, however, assigned less than half of the samples seropositive and the correlation between the Multiplex and tested ELISA assays was poor. Earlier the Multiplex assay has been tested to give comparable results with GST capture ELISA (Waterboer et al., 2005). Antigen in current ELISA assays were *E. coli* expressed His-tagged L1 protein or an unknown antigen thus it may be that the epitopes differ from each other and from the GST-tagged pentameric L1 protein used in Multiplex assay. The lateral flow assay, instead, had VLPs as antigen and correlated best with the Multiplex assay, but apparently being less sensitive. The lateral flow assay reacted with the serum samples that gave the highest response in the Multiplex assay. The biological meaning of such high antibody response remains to be elucidated.

In conclusion, we have shown that anti HPV16L1 IgA and IgG antibodies are detectable in saliva by Multiplex and ELISA assays. Different HPV16L1 antibody assays may expose different epitopes, even though using the same antigen, and therefore the comparison of results from different assays should be done with care. The total antibody concentrations in saliva vary significantly between individuals and therefore measuring only the specific HPV16 responses in saliva can lead to wrong conclusions. Furthermore, salivary HPV16L1 antibody reactivity appears not to correlate with serum HPV16L1 antibodies in men. Thus, measuring salivary antibodies and defining relevant negative controls are of importance in order to gain understanding of biology of and host responses to oral HPV infections.

Conflict of interest

None.

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