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Heparin-based ELISA reduces background reactivity in virus-like particle-based papillomavirus serology

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The interaction between human papillomavirus (HPV) particles and cell surface heparan sulfate requires intact conformation of the HPV particles. Type-specific HPV serology is currently based on virus-like particles (VLPs) with intact conformation. Presence of incorrectly folded VLPs in VLP preparations is recognized as an important cause of cross-reactivity in HPV serology. Heparin-coated microtitre plates were evaluated for capturing conformationally correct VLPs and improving the type specificity of HPV serology. Hybrid VLPs between HPV16 and HPV11, which had been found to have significant reactivity with children's sera and a batch of HPV18 VLPs that had failed the quality control because of significant reactivity with sera from virginal women, were tested in parallel with heparin ELISA, ordinary ELISA and type-specific mAb capture ELISA. Control sera from children that had detectable reactivity with HPV16/11 hybrid VLPs in ordinary ELISA did not react in heparin-based ELISA, but some hybrid VLPs also had background reactivity in capture ELISAs. Control sera from virginal women that had some reactivity with a poor quality HPV18 VLP preparation in ordinary ELISA had no reactivity in heparin or capture ELISA, suggesting that certain VLP preparations expose cross-reactive epitopes that are not exposed on VLPs with heparin-binding ability. As the sensitivity was similar or only marginally affected by the use of heparin plates, use of heparin-coated plates may improve the type specificity of VLP-based ELISAs and reduce interassay variability attributable to variable quality of different VLP batches.

INTRODUCTION

Genital human papillomavirus (HPV) infections are the most common viral sexually transmitted diseases worldwide and have been found to be the central aetiological risk factor for cervical cancer (Schiffman *et al.*, 1993; Walboomers *et al.*, 1999). The major capsid protein L1 of HPV can self-assemble into virus-like particles (VLPs), which are morphologically and immunologically similar to native virions (Kirnbauer *et al.*, 1992; Rose *et al.*, 1993). VLPs are useful tools to evaluate humoral responses to HPV and to investigate early events in infectivity such as cell binding and internalization (Galloway & McDougall, 1996; Muller *et al.*, 1995; Roden *et al.*, 1994; Volpers *et al.*, 1995) and have been used to successfully vaccinate humans against HPV infection (Koutsky *et al.*, 2002). Studies using papillomavirus neutralizing monoclonal antibodies (N-mAbs) have demonstrated that the predominant neutralizing epitopes are type-specific surface conformational epitopes on intact virions or VLPs (Christensen *et al.*, 1990; Ludmerer *et al.*, 1996; Roden *et al.*, 1997). There is a need to define quality control methods, both for HPV serology and vaccinology, that will detect if the virus-like particle (VLP) batches produced have an intact conformation with appropriate exposure of type-specific neutralizing epitopes and minimal exposure of broadly cross-reactive, non-neutralizing epitopes.

As HPVs do not grow efficiently in cell culture, systems using HPV VLPs have been used to study viral infectivity (Roden *et al.*, 1996; Rossi *et al.*, 2000; Stauffer *et al.*, 1998; Unckell *et al.*, 1997). Cell surface heparan sulfates are essential for infection by HPV16 and HPV33 pseudovirions (Giroglou *et al.*, 2001) as well as HPV11 virions (Shafti-Keramat *et al.*, 2003) and for binding and uptake of VLPs of various HPV-types (Combita *et al.*, 2001; Drobni *et al.*, 2003; Joyce *et al.*, 1999; Selinka & Sapp, 2003). Heparin, a highly sulfated form of heparan sulfate proteoglycan secreted by mast cells, interferes with pseudoinfection and has been shown to efficiently bind VLPs (Giroglou *et al.*, 2001; Joyce *et al.*, 1999). The interaction with heparin requires intact conformation of VLPs (Giroglou *et al.*, 2001; Rommel *et al.*, 2004).

VLP-based serology is of widespread importance for studies of HPV epidemiology and vaccinology. VLP ELISAs have been successfully used in epidemiological studies of HPV infections in different populations (Dillner, 1999). However, incorrectly folded VLPs expose cross-reactive epitopes that may compromise the type specificity of VLP-based HPV serological assays (Wang *et al.*, 1997a), a problem recently highlighted in a study with HPV16/11 hybrid VLPs (Wang *et al.*, 2003).

An alternative method to avoid background caused by incorrectly folded VLPs is to use type-specific mAbs against conformational epitopes as catching antibodies in the ELISAs (Carter *et al.*, 1994). However, we have previously found that adequate isotype specificity of ELISAs is improved by using mAbs specific for different human Ig isotypes (Heino *et al.*, 1995; Wang *et al.*, 2000). Also, two-step detection of bound antibodies (with a mouse mAb and an anti-mouse reagent was important for sensitivity (Heino *et al.*, 1995). Two-step methods cannot be used if ELISA plates have been coated with mouse mAbs. Finally, type-specific mAbs to HPV are only available for a limited number of HPV-types.

In the present study, we evaluated whether heparin-coated plates could be used to improve performance of VLP serology. HPV16/11 hybrid VLPs that had significant reactivity with children's sera and a batch of HPV18 VLPs that had failed the quality control because of significant reactivity with sera from virginal women were tested in parallel with heparin-based ELISA, direct ELISA and type-specific mAb capture ELISA using positive- and negative-control validation panels of serum samples.

METHODS

Preparation of heparin/BSA-coated plates. Heparin/BSA-coated plates were prepared by coating microtitre plates (Polysorb; Nunc) with heparin/BSA (1 μg ml^{-1} in PBS, 100 ng per well; Sigma) overnight at room temperature. Plates were subsequently washed three times with 150 μl PBS/0·1 % Tween 20 (PBS-T). Free binding sites were blocked with 150 μl BSA (100 μg ml^{-1}) in PBS-T for 3 h at room temperature. Plates were again washed three times with PBS-T, once with distilled water and subsequently dried by vacuum. Plates were individually sealed with plastic plate sealing tape and stored at 4 °C. Reactivity of the plates did not change over a monitored time span of 18 months.

Serum samples. Serum samples that were HPV16+/HPV11− or HPV16−/HPV11+ were selected from three previous serological studies. Study 1 investigated attack rates and seroprevalences of human papillomavirus types 11, 16 and 33 among a high-risk population for cervical cancer: in the Shanxi province, China. In total, 1196 high school students/teachers (570 men and 626 women, mean age 16 years, range 14–35 years) and 170 couples (mean age 24 years, range 20–35 years) attending the mandatory serological screening before marriage donated serum samples at enrolment and 12 months later. Study 2 investigated the human papillomavirus antibody response among 216 women (mean age 53 years, range 23–88 years) with untreated primary invasive cervical cancer admitted to the Karolinska Hospital, Stockholm, Sweden. Study 2 also contained 243 age and sex-matched healthy control women (Wang *et al.*, 1997b). Study 3 investigated sexual behaviour and the risk of HPV infection in 188 serum samples from 94 teenage girls (mean age 16·1 years) donated at enrolment and at a follow-up 2 years later. Forty-five samples were from virgins, whereas the other samples were from sexually experienced girls. None of the virgin girls had HPV DNA in the cervix (by PCR) or serum antibodies against HPV16 or HPV33 (Andersson-Ellstrom *et al.*, 1996). HPV16+/HPV11− and HPV16−/HPV11+ samples from these studies were re-tested with HPV16 and HPV11 in parallel with the hybrid VLPs. Besides these, children's serum samples were selected from a study that surveyed the seroprevalence of human papillomavirus types 16, 18 and 33 among 1031 children up to 13 years of age, whose serum samples had been submitted for serological analyses of non-HPV-related diseases and were obtained from the Department of Clinical Virology of the Karolinska Hospital and the Department of Pediatrics of the Huddinge Hospital, both in Stockholm, Sweden (af Geijersstam *et al.*, 1999). Serum samples from children in the age group 2–10 years that had been found to be HPV16 seronegative were also tested with HPV11 and re-tested with HPV16 in parallel with the hybrid VLPs to ensure that they were both HPV16 and HPV11 seronegative. In total, 32 HPV16+/HPV11− serum samples, 64 HPV16−/HPV11+ serum samples and 23 HPV16−/HPV11− serum samples were included in this study. In the case of HPV18, serum samples from 13 women who had tested HPV18 DNA+ by PCR of cervical samples and seven serum samples from HPV18 DNA− virginal women were analysed.

ELISA methods. In heparin-based ELISA, intact HPV16, HPV11 capsids and hybrid VLPs H11:16[172–505] (contains the N-terminal part of HPV11 and the C-terminal part of HPV16), H11:16 [FG+HI] (two non-contiguous hypervariable regions of HPV16 L1 containing the FG and HI loops transplanted into HPV11 L1 backbone) and H16:11[171–505] (contains the N-terminal part of HPV16 and the C-terminal part of HPV11) were coated onto heparin-BSA microtitre plates in PBS at pH 7·2. The coating concentrations of VLPs were normalized as described (Wang *et al.*, 2003). The low quality HPV18 batch was coated at 1 μg ml^{-1}.

For generation of disrupted VLPs, three different disruption methods were used: (i) alkali treatment: the VLPs were incubated for 4 h at room temperature in 0·1 M carbonate buffer (pH 9·6); (ii) alkali and reducing agent treatment: the VLPs were incubated for 30 min at room temperature in 0·2 M Na_2CO_3 (pH 10·6) and 0·01 M DTT; (iii) boiling, alkali and reducing agent treatment: the VLPs were heated for 15 min at 96 °C in 0·2 M Na_2CO_3 (pH 10·6) and 0·01 M DTT. Disrupted bovine papillomavirus (BPV) capsids were used as a negative control in all assays.

After 1 h at 37 °C, plates were washed three times with 150 μl PBS-T and blocked by adding 150 μl 10 % horse serum in PBS (HS-PBS) at 37 °C for 15 min. Serum samples were added in duplicate at a 1:30 dilution in HS-PBS for 1 h reaction at 37 °C. Six wells in each plate were incubated with only 10 % HS-PBS. Following three washes with PBS-T, a mAb to human IgG, 1:800 diluted in 10 % HS-PBS, was added. The mAb positive controls (V5 at a 1:1 000 000 dilution, H11.F1 at a 1:100 000 dilution, and H11.H3 at a 1:10 000 dilution) were added to the wells that had been incubated only with HS-PBS. The plates were incubated for 45 min at 37 °C. After washing three times, a goat anti-mouse IgG HRP conjugate, 1:2000 diluted in HS-PBS, was added for 30 min at 37 °C, followed by the addition of a substrate. The absorbance of each serum with disrupted BPV VLPs was subtracted from the HPV VLP reactivity and the mean value of the duplicates was calculated.

For estimation of antibody levels in units, the serum samples were tested in duplicate with three different dilutions (1:10, 1:31·6, 1:100), the mAb to human IgG 1:500 diluted in 10 % HS-PBS

was added, and the plates were read after 60 min. The absorbance of each serum with disrupted BPV was subtracted from the VLP reactivity, the mean value of the duplicates was calculated, and then the OD values were transformed into ELISA units using the PLL (parallel line) method (Grabowska *et al.*, 2002).

For capture ELISAs, the HPV16 mAb V5 diluted 1 : 1000 in cold PBS (pH 7·2) or the HPV18 N-mAb R5, diluted 1 : 2000, were coated onto microtitre plates overnight. After the plates were washed once with PBS-T and blocked with 10 % HS-PBS for 1 h at room temperature, VLPs were added for 2 h at room temperature. Following five thorough washings of the plates, serum samples were added either in duplicate at a 1 : 30 dilution or or in duplicate at 1 : 10, 1 : 31·6 and 1 : 100 dilutions for 2 h at room temperature. A rabbit anti-human IgG HRP conjugate (DAKO) was diluted 1 : 1000 in HS-PBS with normal mouse serum (25 μl ml^{-1}) and rotated end-over-end for 4 h. In-between washing of the plates, the conjugate was added to the plates and incubated for 1 h at room temperature, followed by addition of substrate. Plates handled identically, except that no VLPs were added, were used as background.

RESULTS

Reactivity of intact and disrupted hybrid VLPs in direct and heparin-based ELISAs

Intact and alkali-disrupted VLPs of HPV16 and HPV11 as well as the three hybrid VLPs between these two viruses were tested for reactivity with mAbs V5 (HPV16 neutralizing, type-specific, conformation-dependent), F1 and H3 (both are HPV11 neutralizing, type-specific, conformation-dependent, but recognize different epitopes), H16.D9 (cross-reactive, non-conformational, non-neutralizing, exposed on disrupted or incorrectly folded VLPs) and H16.B20 (strongly reactive with both intact and disrupted HPV16, non-neutralizing) in direct and heparin-based ELISAs (Table 1).

The type-specific N-mAbs reacted similarly with intact hybrid VLPs in heparin-based and direct ELISAs, although reactivity was usually somewhat lower in heparin-based ELISA. The reactivity of mAb H16.D9 to intact VLPs was also similar in heparin-based and direct ELISAs. In contrast, the reactivity of H16.D9 with disrupted virus was eliminated or strongly reduced on heparin-coated plates. Indeed, none of the mAbs that reacted with disrupted virus in direct ELISAs were reactive with the same disrupted viruses if heparin plates were used, with the exception of the reactivity of disrupted H11 : 16[FG + HI] hybrid virus with the H16.D9 mAb. The remaining H16.D9 reactivity in heparin-based ELISA suggested that some intact VLP might be present in the alkali-disrupted H11 : 16[FG + HI] hybrid VLPs. Therefore, we also examined more harsh methods for disruption of VLPs (alkali treatment and reducing agent treatment; boiling, alkali and reducing agent treatment). With the most harsh method (boil/alkali/reducing agent), there was no antigenic reactivity at all detectable on the heparin-coated plates, although antigenic reactivity with similarly disrupted VLPs was readily detectable on ordinary ELISA plates (Table 1).

Reactivity of HPV16/11 hybrid VLPs with human serum samples in heparin-based and capture ELISAs

Thirty-two HPV16 + /HPV11 − serum samples, 64 HPV16 − /HPV11 + serum samples and 23 HPV16 − / HPV11 − children's serum samples were tested in heparin-based ELISA in comparison with direct ELISA for reactivity with HPV16 and HPV11 VLPs and with the three hybrid VLPs. Similar experiments with direct ELISAs have been published previously (Wang *et al.*, 2003).

The antibody responses among HPV16 − /HPV11 + human sera to hybrid VLPs were rather similar in heparin-based and direct ELISAs (Table 2). The HPV11 + / HPV16 − sera had very similar reactivity with HPV11 and hybrids that contained the HPV11 C terminus (H11 : 16[FG + HI] and H16 : 11[171–505]) (Fig. 1). There were minor differences in correlation depending on whether heparin-based or direct ELISAs had been used. For example, H11 : 16[FG + HI] versus HPV11 had a correlation coefficient in direct ELISA of 0·58 (Wang *et al.*, 2003), in heparin-based ELISA of 0·68. The correlation coefficient between hybrid H16 : 11 [171-505] to HPV11 was 0·71 for direct ELISA (Wang *et al.*, 2003) and 0·63 for heparin-based ELISA (Fig. 1).

In previous studies (Wang *et al.*, 2003) we found that there was a remarkable difference in the level of antibody reactivity for three hybrid VLPs among HPV16 − /HPV11 + human sera, with H11 : 16[FG + HI] giving on average almost three times higher reactivity than HPV11, although similar amounts of VLPs (titrated to be optimal concentrations of VLP antigen) were used in each ELISA assay. We suggested that this increased reactivity would be due to subtle differences in surface exposure of conformationally constrained epitopes on hybrid VLPs. It was therefore of interest to study whether this increased reactivity would also be present if heparin-based ELISAs was used. The antibody responses among HPV16 − /HPV11 + sera to the hybrid VLPs were quite similar in heparin-based and direct ELISAs (for H11 : 16[FG + HI], mean OD value 1·48 in direct ELISA and 1·41 in heparin-based ELISA). Hybrid H11 : 16[FG + HI] still gave more than two times higher reactivity than HPV11 VLP (Table 2).

The level of the antibody responses among HPV16 + / HPV11 − sera to all three hybrid VLPs were remarkably decreased and the correlation of the reactivity of HPV16 + / HPV11 − human sera against HPV16 as compared with the reactivity against all three hybrids to HPV16 was lower in heparin-based ELISA than in direct ELISA (Table 2). For example, H11 : 16[172–505] versus HPV16 had a correlation coefficient in direct ELISA of 0·78 (Wang *et al.*, 2003), in heparin-based ELISA of 0·60 (Fig. 2).

Interestingly, all HPV16 − /HPV11 − control sera from children that had detectable reactivity with hybrid VLPs in direct ELISAs were no longer positive in the heparin-based ELISA (Tables 2 and 3). All children's sera had OD

Table 1. Reactivity with mAbs in direct and heparin-based ELISAs

Hybrid VLP H11 : 16[172–505] contains the N-terminal part of HPV11 and the C-terminal part of HPV16; hybrid VLP H16 : 11[171–505] contains the N-terminal part of HPV16 and the C-terminal part of HPV11; hybrid VLP H11 : 16[FG + HI] contains two non-contiguous hypervariable regions of HPV16 L1 containing the FG and HI loops transplanted into HPV11 L1 backbone. DE, Direct ELISA; HE, heparin-based ELISA.

mAb	VLP antigen (ELISA absorbance)																			
	HPV16				HPV11				H11 : 16[172–505]				H11 : 16[FG + HI]				H16 : 11[171–505]			
	Intact		Disrupted*		Intact		Disrupted*		Intact		Disrupted*		Intact		Disrupted*		Intact		Disrupted*	
	DE	HE	DE	HE	DE	HE	DE	HE	DE	HE	DE	HE	DE	HE	DE	HE	DE	HE	DE	HE
(a) Reactivity of intact and alkali-disrupted VLPs																				
H16.D9	0·418	0·492	0·604	0·035	0·061	0·211	1·031	0·101	0·161	0·174	0·281	0·031	1·211	1·764	2·420	0·523	0·705	1·326	1·850	0·070
H16.B20	1·821	1·168	2·427	0·079	0·077	0·057	0·201	0·023	0·470	0·574	1·541	0·097	0·657	0·292	2·174	0·064	0·286	0·218	0·543	0·042
H16.V5	2·440	2·445	0·031	0·004	0·004	0·000	0·001	0·000	1·886	2·198	0·167	0·011	2·440	0·814	0·605	0·004	0·020	0·017	0·016	0·008
H11.F1	0·020	0·012	0·033	0·006	2·438	2·443	2·431	0·000	0·299	0·083	0·031	0·000	2·438	1·292	0·220	0·000	0·027	0·023	0·033	0·000
H11.H3	0·029	0·022	0·029	0·013	2·061	1·220	1·862	0·000	0·041	0·005	0·031	0·007	0·036	0·000	0·027	0·008	1·947	1·108	0·035	0·020
(b) Reactivity of reducing agent-disrupted VLPs																				
	Disrupted†		Disrupted‡		Disrupted†		Disrupted‡		Disrupted†		Disrupted‡		Disrupted†		Disrupted‡		Disrupted†		Disrupted‡	
	DE	HE	DE	HE	DE	HE	DE	HE	DE	HE	DE	HE	DE	HE	DE	HE	DE	HE	DE	HE
H16.D9	0·280	0·011	0·294	0·000	0·369	0·021	0·244	0·000	0·087	0·037	0·008	0·011	1·376	0·252	0·996	0·013	0·632	0·046	0·337	0·001
H16.B20	2·282	0·080	2·408	0·021	0·156	0·008	0·299	0·000	0·468	0·053	0·729	0·011	1·352	0·030	1·237	0·000	0·345	0·010	0·369	0·000
H16.V5	0·005	0·012	0·000	0·000	0·005	0·003	0·003	0·000	0·054	0·053	0·000	0·039	0·030	0·019	0·002	0·006	0·019	0·010	0·000	0·002
H11.F1	0·002	0·000	0·000	0·000	0·004	0·000	0·001	0·000	0·059	0·037	0·000	0·022	0·026	0·017	0·000	0·007	0·022	0·009	0·000	0·000
H11.H3	0·001	0·000	0·002	0·000	0·000	0·000	0·005	0·000	0·052	0·006	0·000	0·023	0·022	0·000	0·000	0·007	0·016	0·000	0·000	0·002

*Disruption by alkali was by incubating VLPs for 4 h at room temperature in 0·1 M carbonate buffer (pH 9·6).

†Disruption with alkali and reducing agent treatment was by incubation for 30 min at room temperature in 0·2 M Na_2CO_3 (pH 10·6) and 0·01 M DTT.

‡Disruption with boiling, alkali and reducing agent treatment was by heating for 15 min at 96 °C in 0·2 M Na_2CO_3 (pH 10·6) and 0·01 M DTT.

Table 2. Comparison of reactivity (mean ELISA absorbance value) of HPV16/11 hybrid VLPs with human serum samples in direct and heparin-based ELISA

The HPV16, HPV11 and the HPV16/11 hybrid VLPs were tested in direct and heparin-based ELISA for reactivity with 32 HPV16+/HPV11− human serum samples, 64 HPV16−/HPV11+ human serum samples and 23 HPV16−/HPV11− control serum samples from children. The table shows the mean ELISA absorbance values and the standard deviations in parentheses for each serum sample group.

Human serum samples	Direct ELISA					Heparin-based ELISA				
	VLP antigen									
	HPV16	HPV11	H11:16 [172–505]	H11:16 [FG+HI]	H16:11 [171–505]	HPV16	HPV11	H11:16 [172–505]	H11:16 [FG+HI]	H16:11 [171–505]
HPV16−/HPV11+	0·031 (0·017)	0·563 (0·197)	0·083 (0·072)	1·480 (0·405)	0·677 (0·256)	0·021 (0·020)	0·645 (0·264)	0·084 (0·102)	1·410 (0·449)	0·672 (0·321)
HPV16+/HPV11−	0·518 (0·309)	0·058 (0·027)	0·208 (0·186)	0·301 (0·167)	0·141 (0·139)	0·466 (0·379)	0·029 (0·022)	0·111 (0·162)	0·103 (0·104)	0·029 (0·060)
HPV16−/HPV11−	0·028 (0·014)	0·046 (0·023)	0·060 (0·024)	0·145 (0·117)	0·068 (0·037)	0·019 (0·019)	0·015 (0·016)	0·022 (0·017)	0·015 (0·023)	0·010 (0·015)

values below 0·1 in the heparin-based ELISA. The 11/23 children's samples that had highest reactivity in the direct ELISA were also tested for the reactivity with the hybrid VLPs H11:16[172–505] and H11:16[FG+HI] containing the V5 epitope in a capture ELISA using the V5 mAb as capture. The children's reactivities in the capture ELISA were quite similar to the ordinary ELISA (Table 3).

Reactivity of low quality HPV18 VLPs in heparin-based, direct and capture ELISAs

Production of high quality, conformationally correct VLPs is particularly difficult for HPV18, with the result that many batches of HPV18 VLPs fail the quality control and cannot be used for serology. Because of the improved specificity of heparin-based ELISA, we also evaluated the reactivity of a batch of HPV18 VLPs that had previously failed in the quality control testing because of measurable reactivity with serum samples from unexposed women. In direct ELISAs, one negative control sample from a virginal woman had strong reactivity with this HPV18 batch and the other samples in the negative control panel also had some detectable reactivity in direct ELISA (Table 4). When this HPV18 VLP batch was tested with mAbs, the expected strong, type-specific reactivity with the HPV18 N-mAb R5 was detected (Table 5). However, weak reactivities with H16.V5, H11.F1 and H11.H3 (type-specific for HPV16 and 11) were also detectable (Table 5). However, no reactivity was detectable when the same negative control sera and mAbs against other HPV-types were tested with the same VLPs coated onto heparin-binding plates (Tables 4 and 5). Thirteen serum samples from HPV18 DNA+ women and seven samples from HPV18 DNA− virginal women were also tested for reactivity with this low-quality HPV18 VLP with capture ELISA using the HPV18 type-specific mAb R5 as a capture molecule. The reactivities were quite similar to those in heparin-based ELISA (Table 4). Both methods had lower background with serum samples from unexposed women and essentially similar reactivity with the samples from infected women (Table 4).

DISCUSSION

In the present study we found that all HPV16−/HPV11− control serum samples from children, which had serological reactivity with HPV16/11 hybrid VLPs in direct ELISAs, were no longer reactive with the same VLPs in heparin-based ELISA, suggesting that non-type-specific serological reactivity to certain VLP preparations may be due to exposure of cross-reactive epitopes that are not exposed on intact, heparin-binding VLPs. Also, the reactivity of serum samples from virginal women with low-quality HPV18 VLPs in direct ELISA was not detectable when the same VLPs were coated onto heparin-binding plates, implying that use of heparin-coated plates may improve HPV type specificity of VLP-based ELISAs and reduce interassay variability attributable to variable quality of VLP batches.

The HPV11+/HPV16− human sera had very similar

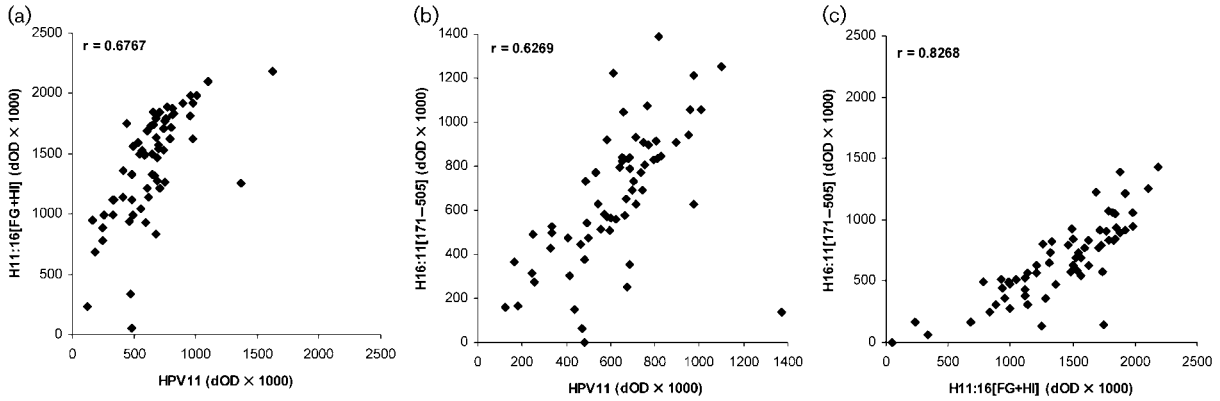

Fig. 1. Reactivity of HPV16/11 hybrid VLPs with HPV16−/HPV11+ human serum samples in heparin-based ELISAs. (a) Hybrid VLP H11 : 16[FG + HI] versus HPV11. (b) Hybrid VLP H16 : 11[171–505] versus HPV11. (c) Hybrid VLPs H11 : 16[FG + HI] versus H16 : 11[171–505]. dOD, Difference in OD between plates coated with specific antigen and plates coated with control antigen.

reactivity with HPV11 and HPV16/11 VLP hybrids that contained the HPV11 C terminus (H11 : 16[FG + HI] and H16 : 11[171–505]) in both heparin-based and direct ELISAs, suggesting that the major human serum-reactive HPV11 epitope(s) is present in these VLPs and that VLPs containing this epitope are heparin-binding.

In contrast, the data for HPV16 were much less clear. The reactivities were much lower in the heparin-based ELISAs and showed lower correlations with the reactivities against the parent virus when heparin-based ELISAs were used. The hybrid virus H11 : 16[FG + HI] was unique in that adequate disruption appeared to require exceptionally harsh disruption methods. Also, this VLP was the only one from which the results of heparin-based and capture ELISAs where distinctly different. Capture ELISAs did not improve background reactivity with children's sera, whereas heparin ELISA did. Conceivably, the artificial addition of the V5-binding site to an HPV11 backbone may have also resulted in the V5 epitope being exposed on incorrectly folded H11 : 16[FG + HI] VLPs.

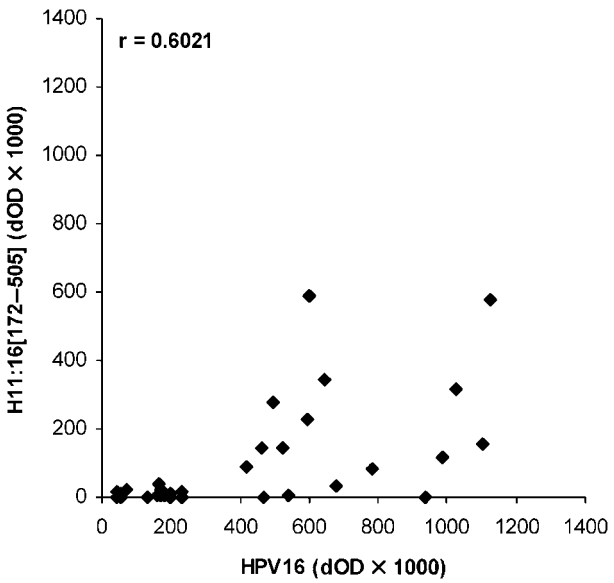

Fig. 2. Reactivity of HPV16/11 hybrid VLP H11 : 16[172–505] versus HPV16 with HPV11−/HPV16+ human serum samples in heparin-based ELISA. dOD, Difference in OD between plates coated with specific antigen and plates coated with control antigen.

Quality control of ELISAs depends on maintaining intact VLPs of good quality and a quality assurance system. We found that the mAbs that reacted with disrupted virus in direct ELISAs did not react with the same disrupted viruses if heparin-coated plates were used, suggesting that the use of heparin-coated microtitre plates could add a quality control component to the VLP-based ELISAs by preventing binding of denatured L1 antigen and contaminating protein. Numerous approaches towards decreasing non-specific binding to ELISA plates have been described, notably the use of blocking with vinyl polymers (Studentsov *et al.*, 2002). The approach with heparin-coated plates has the advantage that a specific biological property of the virus is exploited to increase the specificity of binding. VLPs of all HPV-types tested so far bind to heparin with similar efficiency (Rommel *et al.*, 2004), suggesting that heparin can be used as a universal capture molecule for HPV serology.

In summary, heparin-coated plates will select a subset of VLPs with intact heparin-binding activity. At least for some HPVs, intact heparin-binding ability was found to be correlated with serological specificity, allowing for a straightforward and simple improvement of the performance of HPV serology.

Table 3. Eleven children's samples with high reactivity in direct ELISA, tested in parallel with heparin-based ELISA and V5-catching antibody ELISA

Sample no.	Direct ELISA		Heparin ELISA		V5-catching antibody ELISA	
	H11 : 16 [172–505]	H11 : 16 [FG + HI]	H11 : 16 [172–505]	H11 : 16 [FG + HI]	H11 : 16 [172–505]	H11 : 16 [FG + HI]
1	0·095	0·265	0·022	0·000	0·047	0·176
2	0·058	0·143	0·000	0·000	0·048	0·089
3	0·053	0·393	0·012	0·040	0·069	0·286
4	0·038	0·354	0·019	0·084	0·087	0·278
5	0·029	0·143	0·028	0·016	0·132	0·158
6	0·043	0·370	0·018	0·030	0·030	0·207
7	0·051	0·133	0·035	0·000	0·052	0·109
8	0·060	0·239	0·020	0·069	0·066	0·139
9	0·032	0·179	0·019	0·023	0·068	0·163
10	0·113	0·263	0·000	0·000	0·125	0·280
11	0·080	0·194	0·009	0·009	0·037	0·208

Table 4. Levels of antibodies against a low-quality HPV18 VLP batch in direct ELISA, in heparin-based ELISA and in catching antibody ELISA

Serum samples from 13 women who had tested HPV18 DNA+ by PCR of cervical samples and seven serum samples from HPV18 DNA− virginal women were tested in direct ELISA, heparin-based ELISA and R5-catching ELISA with a low-quality HPV18 VLP batch. Antibody levels are expressed as ELISA units relative to a reference standard serum. Cut-off for positivity is 0·54 ELISA units for all assays and positive sera are marked in bold.

	Sample no.	Direct ELISA	Heparin ELISA	R5-catching antibody ELISA
HPV18 DNA+ women	1	**7·39**	**7·15**	**9·55**
	2	**1·40**	**1·55**	**2·38**
	3	**3·64**	0·14	0·16
	4	**2·18**	**1·81**	**2·52**
	5	**1·41**	**1·26**	**1·83**
	6	**1·15**	**0·97**	**1·18**
	7	0·08	0·04	0·13
	8	0·19	0·03	0·23
	9	0·14	0·09	0·26
	10	**1·15**	**0·78**	**1·29**
	11	**1·67**	**1·73**	**1·31**
	12	0·03	0·11	0·13
	13	0·05	0·00	0·03
Virginal women	14	0·07	0·00	0·04
	15	**1·03**	0·00	0·05
	16	0·10	0·00	0·10
	17	0·09	0·00	0·07
	18	0·15	0·02	0·03
	19	0·12	0·00	0·00
	20	0·29	0·00	0·03

Table 5. Reactivity of intact and disrupted low-quality HPV18 VLPs with mAbs

DE, Direct ELISA; HE, heparin-based ELISA.

mAb	HPV18 VLP (ELISA absorbance)							
	Intact		Disrupted*		Disrupted†		Disrupted‡	
	DE	HE	DE	HE	DE	HE	DE	HE
H18.R5	2·118	1·584	1·379	0·244	2·318	0·000	0·019	0·000
H16.D9	0·042	0·014	0·029	0·015	0·008	0·000	0·032	0·004
H16.B20	0·025	0·016	0·018	0·005	0·007	0·032	0·028	0·000
H16.V5	0·478	0·034	0·225	0·005	0·003	0·000	0·027	0·000
H11.F1	0·144	0·021	0·075	0·004	0·004	0·001	0·027	0·003
H11.H3	0·269	0·009	0·123	0·009	0·061	0·000	0·008	0·000

*Disruption by alkali was by incubating VLPs for 4 h at room temperature in 0·1 M carbonate buffer (pH 9·6).

†Disruption with alkali and reducing agent treatment was by incubation for 30 min at room temperature in 0·2 M Na_2CO_3 (pH 10·6) and 0·01 M DTT.

‡Disruption with boiling, alkali and reducing agent treatment was by heating for 15 min at 96 °C in 0·2 M Na_2CO_3 (pH 10·6) and 0·01 M DTT.

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