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Assessment of HPV 16 and HPV 18 antibody responses by pseudovirus neutralization, Merck cLIA and Merck total IgG LIA immunoassays in a reduced dosage quadrivalent HPV vaccine trial

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ABSTRACT

We assessed HPV 16 and 18 antibody responses of female subjects enrolled in a 2- vs. 3-dose quadrivalent HPV (Q-HPV) vaccine trial (ClinicalTrials.gov NCT00501137) using the Merck competitive Luminex (cLIA) and total IgG Luminex (TIgG) immunoassays, and a pseudovirus neutralizing antibody (PsV NAb) assay. Subjects were enrolled in one of three groups: (1) 9–13 yr, 2 doses of Q-HPV at 0, 6 months ($n = 259$); (2) 9–13 yr, 3 doses at 0, 2, 6 months ($n = 260$); and (3) 16–26 yr, 3 doses at 0, 2, 6 months ($n = 305$). Sera were collected from all subjects at baseline, months 7 and 24, and from half the subjects at months 18 and 36. High correlation was observed between all three assays. At month 36, HPV 16 antibodies remained detectable in all subjects by all assays, whereas 86.4%, 99.6% and 100% of subjects respectively were HPV 18 cLIA, TIgG and PsV NAb (partial neutralization endpoint) seropositive. The proportion seropositive for HPV 18 by cLIA at 36 months was not significantly different for 2-dose girls vs. 3-dose adults (85.9% vs. 79.4%; $p = 0.51$), whereas the proportion for 3-dose girls was significantly higher than for 3-dose adults (95.3% vs. 79.4%; $p < 0.01$). The HPV 18 seropositive proportions by the TIgG and PsV NAb (partial neutralization endpoint) assays were the same for all subjects. High baseline HPV 16 and HPV 18 seropositivity was observed for the TIgG assay and it is unclear if all the detected TIgG antibodies are type-specific and/or neutralizing. For the PsV NAb assay, 90% and partial neutralization geometric mean titres were consistently 2–8-fold higher than for 100% neutralization, which enabled detection of HPV 18 NAb in subjects who lost detectable cLIA antibodies over time. We conclude that the PsV NAb assay is more sensitive than the cLIA, and likely more specific than the TIgG assay.

Abbreviations: cLIA, Merck competitive Luminex immunoassay; EIA, enzyme immunoassay; GMT, geometric mean titre; mMU, milli-Merck units; NT_{100}, 100% neutralization endpoint; NT_{90}, 90% neutralization endpoint; $NT_{partial}$, partial neutralization endpoint; PsV NAb, pseudovirus neutralizing antibody; Q-HPV, quadrivalent HPV; RFP, red fluorescent protein; TIgG, Merck total IgG Luminex immunoassay; VLP, virus-like particle.

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1. Introduction

Human papillomavirus (HPV) vaccines induce type-specific neutralizing antibodies which correlate with immunity to the corresponding HPV types [1], and World Health Organization guidelines recommend that assays which assess neutralization be used as the reference standard for measuring HPV vaccine responses [2]. Quadrivalent HPV (Q-HPV) vaccine (Gardasil®, Merck Laboratories) consists of HPV 6, 11, 16 and 18 virus-like particles (VLP) and is licensed for a 3-dose regimen. Post-Gardasil® antibody responses are typically measured by a proprietary multiplex competitive Luminex immunoassay (cLIA) [3], which is based on competitive binding of type-specific HPV antibodies in human sera with labelled monoclonal antibodies directed against neutralizing epitopes of the respective VLP types (HPV 6, 11, 16 and 18). It has been reported that HPV antibodies measured by the cLIA may decline to become undetectable over time, especially for HPV 18, despite continued vaccine efficacy in preventing infections [4,5]. The significance of the loss of detectable antibodies is unknown as protective levels of HPV antibodies remain undefined [1,6,7] and vaccine efficacy remains near 100%. Recently, Merck Laboratories developed a total IgG Luminex immunoassay (TIgG) which measures antibodies against the entire VLP, i.e., a broader array of VLP antibodies than the cLIA, for nine HPV types (HPV 6, 11, 16, 18, 31, 33, 45, 52 and 58) [8].

In order to further characterize HPV antibody responses in a 2- vs. 3-dose randomized controlled Q-HPV vaccine trial, we adapted and implemented the National Institutes of Health pseudovirus neutralizing antibody (PsV NAb) assay [9], in which a red fluorescent protein (RFP) reporter plasmid was incorporated into the PsV [10]. Neutralizing antibodies block PsV entry into susceptible cells and prevent expression of the RFP which is visualized by fluorescence microscopy. While PsV NAb assays are technically complex and have not been standardized, they provide an alternative to vaccine manufacturers' assays by detecting type-specific antibodies that block HPV infection of susceptible cells.

We previously reported HPV 16 and 18 PsV NAb and cLIA responses for the 2- vs. 3-dose trial at 7 months post-vaccination [11]. We now report HPV 16 and HPV 18 PsV NAb, Merck cLIA and Merck TIgG antibody responses through to 36 months post-vaccine.

2. Materials and methods

2.1. Study population

The study population consisted of 824 females aged 9–26 years at three study sites in Canada (British Columbia, Québec and Nova Scotia), who were enrolled into one of three study arms as previously described [12]. Younger subjects (9–13 yr) were randomly assigned to receive two or three doses of Q-HPV vaccine, whereas older subjects (16–26 yr) received only the standard three dose regimen. Distribution among the study arms was: Group 1 (n = 259), 9–13 yr (mean age 12.4 yr), received two doses at months 0 and 6; Group 2 (n = 260), 9–13 yr (mean age 12.3 yr), received three doses at months 0, 2 and 6; and Group 3 (n = 305), 16–26 yr (mean age 19.3 yr), received three doses at months 0, 2 and 6 (Fig. 1). Sera were collected from the entire cohort at baseline, months 7 and 24; in addition, half the cohort was randomly selected for serum collection at month 18, and the other half had serum collected at month 36. Group 3 subjects also provided self-collected vaginal swabs (HC^{TM} Female Swab Specimen Collection Kit; Qiagen) to determine if HPV 16 or HPV 18 DNA positivity at baseline impacted the respective antibody responses.

Informed consent was obtained for all subjects after explaining the nature and possible consequences of the study. The study was approved by the University of British Columbia Clinical Research Ethics Board and by local research ethics boards at the other sites. The clinical trial was registered with ClinicalTrials.gov (NCT00501137).

2.2. HPV antibody assays

The PsV NAb assay was performed as previously described [10]. Briefly, HPV 16 and 18 PsV incorporating RFP were prepared by transfection of 293TT cells with HPV 16 or 18 L1 and L2 plasmids together with RFP plasmids. PsV preparations were purified and titrated in 293TT cells. The PsV L1 protein concentrations were estimated by comparing polyacrylamide gel electrophoresis L1 band densities for each PsV preparation with the densities of known concentrations of HPV 16 and 18 Merck vaccine VLPs. The HPV 16 PsV contained approximately 2–3-fold more L1 compared to the HPV 18 PsV.

Subject sera were serially diluted, mixed with 100 infectious units of the respective HPV 16 or 18 PsV, and inoculated onto 293TT

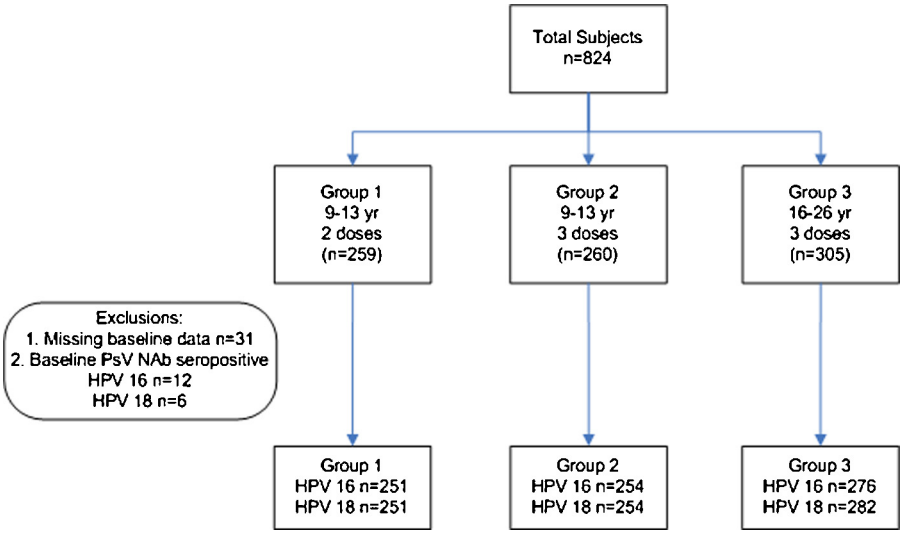

Fig. 1. Distribution of study subjects. Distribution of study subjects by age group and dosing regimen, and description of exclusions. *Abbreviation*: PsV NAb, pseudovirus neutralizing antibody.

cells in microtitre plates. Cultures were monitored by fluorescence microscopy for four to six days. Three serum titration endpoints were defined: NT_{100}, the highest dilution of serum which completely blocked RFP expression (100% neutralization); NT_{90}, the highest dilution which blocked 90% of RFP expression (90% neutralization) and $NT_{partial}$, the highest dilution which partially blocked RFP expression (>10% and <90% neutralization). All sera were tested in duplicate and geometric mean titres (GMT) were determined for each endpoint, except that NT_{90} and $NT_{partial}$ endpoints could not always be determined, e.g., when the dilution following the NT_{100} endpoint showed no neutralization. HPV 16 or 18 PsV NAb seropositivity was defined as a GMT of ≥40 and was determined for each of the NT_{100}, NT_{90} or $NT_{partial}$ endpoints.

Merck cLIA and TIgG testing was performed at Merck Research Laboratories as previously described [8,13]. Geometric mean antibody levels for both assays were expressed as milli-Merck units (mMU) per mL. The cLIA was considered positive if the result was ≥20 mMU for HPV 16 and ≥24 mMU for HPV 18; the TIgG assay was considered positive if the result was ≥7 mMU for HPV 16 and ≥10 mMU for HPV 18. Testing laboratories were blinded to the dosing regimens.

2.3. HPV DNA testing

Self-collected baseline vaginal swab specimens ($n = 303$) from Group 3 subjects were tested for HPV DNA by the Roche Linear Array HPV Genotyping Test (Roche Diagnostics), which detects 37 high- and low-risk HPV types, including HPV 16 and 18.

2.4. Statistical analysis

For the longitudinal antibody response assessments and calculations for assay correlation, eligible subjects were those who had baseline data available for all three assays and who were seronegative for PsV NAb (NT_{100}) at baseline (Fig. 1). Pearson correlation coefficients were calculated for the respective pooled 7-, 18-, 24- and 36-month PsV NAb, cLIA and TIgG antibody levels. Multiple comparisons of the binomial seropositive proportions by study group and antibody assay were performed by the permutation resampling method [14]. The Wilcoxon Rank Sum Test was used to compare the 36-month antibody levels for each of the assays for (1) baseline HPV 16 or 18 seropositive vs. the respective baseline seronegative subjects, and (2) baseline HPV 16 or 18 DNA positive vs. the respective baseline HPV DNA negative subjects. All statistical calculations were performed using SAS v.9.1.3 (Statistical Analysis Software, Cary, NC).

3. Results

Correlations for the PsV NAb, cLIA and TIgG assays are shown in Table 1 and Supplementary Fig. 1. PsV NAb and cLIA correlated more closely for HPV 18 than for HPV 16, whereas the correlation between PsV NAb and TIgG was similar for both HPV 16 and 18.

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2013.09.007.

Table 2 shows the proportions of subjects seropositive for HPV 16 and 18 for the three assays through to 36 months post-vaccine. At baseline, 0.1% of PsV NAb NT_{100} negative subjects were HPV 16 cLIA seropositive and none were HPV 18 cLIA seropositive, whereas 10.8% and 27.5% respectively were baseline TIgG seropositive. At month 36, HPV 16 antibodies remained detectable in all subjects by all three assays. In contrast, beginning at 18 months post-vaccine, HPV 18 antibodies could not be detected by cLIA in a proportion of subjects, and by month 36, 13.6% overall of subjects had no detectable HPV 18 cLIA antibodies. When stratified by study group, HPV 18 cLIA seropositivity at 36 months was 85.9% for 2-dose girls (Group 1), 95.3% for 3-dose girls (Group 2) and 79.4% for 3-dose adults (Group 3) (1 vs. 2 $p = 0.11$; 1 vs. 3 $p = 0.51$; 2 vs. 3 $p < 0.01$). The TIgG assay detected HPV 18 antibodies in most subjects and all subjects were PsV NAb seropositive ($NT_{partial}$ endpoint) at 36 months.

Table 1
Pearson correlation coefficients for PsV NAb, cLIA and TIgG assays.

	HPV 16		HPV 18	
	n	r^2	n	r^2
NT_{100} vs. cLIA	1705	0.862	1611	0.934
NT_{100} vs. TIgG	1705	0.931	1610	0.933
NT_{90} vs. cLIA	1468	0.863	1509	0.939
NT_{90} vs. TIgG	1468	0.923	1508	0.936
$NT_{partial}$ vs. cLIA	1622	0.779	1741	0.901
$NT_{partial}$ vs. TIgG	1622	0.869	1740	0.894
cLIA vs. TIgG	1939	0.865	1953	0.942

Abbreviations: PsV NAb, pseudovirus neutralizing antibody; cLIA, Merck competitive Luminex immunoassay; TIgG Merck total IgG Luminex immunoassay; r^2, Pearson correlation coefficient; NT_{100}, PsV NAb 100% neutralization endpoint; NT_{90}, PsV NAb 90% neutralization endpoint; $NT_{partial}$, PsV NAb partial neutralization endpoint.

HPV 16 NT_{100} GMTs for 2-dose girls were similar to those for 3-dose girls through to 36 months (Table 3), and both 2- and 3-dose girls had HPV 16 NT_{100} GMTs approximately 2- to 3-fold higher than 3-dose adults at all time points. For HPV 18, NT_{100} GMTs were similar for both 2- and 3-dose girls at 7 months, and both groups had higher GMTs than 3-dose adults. At 18, 24 and 36 months, HPV 18 GMTs for 2-dose girls were about 2-fold lower than those for 3-dose girls, but at 36 months, GMTs for 2-dose girls remained similar to those for 3-dose adults. Responses measured by the cLIA and TIgG assays showed similar patterns. NT_{90} and $NT_{partial}$ GMTs for both HPV 16 and 18 were consistently 2- to 8-fold higher respectively than the corresponding NT_{100} GMTs (Table 3 and Supplementary Fig. 2).

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2013.09.007.

There was no statistically significant difference in HPV 16 and 18 antibody levels at 36 months for baseline seropositive vs. baseline seronegative subjects (Table 4), and subjects who were baseline seropositive demonstrated 36 month antibody levels similar to those achieved by baseline seronegative subjects. Baseline HPV 16 DNA positive vs. negative subjects had similar 36 month antibody levels, whereas 36 month antibody levels for HPV 18 DNA positive vs. negative subjects were approximately 2- to 3-fold higher. However, this difference did not achieve statistical significance (Table 5).

4. Discussion

Among subjects enrolled in this 2-dose vs. 3-dose Q-HPV vaccine trial, HPV 16 antibodies measured by the cLIA, TIgG and PsV NAb assays remained detectable for at least 36 months for all subjects. In contrast, beginning at 18 months post-vaccine, the cLIA was unable to detect HPV 18 antibodies in a subset of subjects, while HPV 18 antibodies remained detectable for at least 36 months in most subjects by the TIgG assay and in all subjects by the PsV NAb assay ($NT_{partial}$ endpoint). Other studies have demonstrated that up to 40 percent of vaccinated subjects lose detectable HPV 18 cLIA antibodies over time, but vaccine efficacy in preventing subsequent HPV 18 infection is maintained [4–6]. Consistent with our observations, when such individuals are tested by the TIgG [15] or a PsV NAb assay [16], HPV 18 antibodies remain detectable in the majority of individuals for at least 48 months. We demonstrated that HPV 16 and HPV 18 antibody titres reach a plateau about 18 months post-vaccine for both 2- and 3-dose regimens, and remain essentially

Table 2
HPV 16 and HPV 18 PsV NAb, cLIA and TIgG seropositivity to 36 months post-vaccine.

	%HPV 16 seropositive					%HPV 18 seropositive				
	cLIA	TIgG	NT_{100}	NT_{90}	$NT_{partial}$	cLIA	TIgG	NT_{100}	NT_{90}	$NT_{partial}$
Baseline	0.13	10.8	0.0	0.0	0.0	0.0	27.5	0.0	0.0	0.0
7 months	100.0	99.7	100.0	100.0	100.0	99.8	99.9	100.0	100.0	100.0
18 months	100.0	100.0	100.0	100.0	100.0	90.2	99.7	91.1	95.0	100.0
24 months	100.0	99.8	99.8	100.0	100.0	88.0	99.2	90.4	93.9	100.0
36 months	100.0	100.0	100.0	100.0	100.0	86.4	99.6	85.3	90.4	100.0

Abbreviations: PsV NAb, pseudovirus neutralizing antibody; cLIA, Merck competitive Luminex immunoassay; TIgG, Merck total IgG Luminex immunoassay; NT_{100}, PsV NAb 100% neutralization endpoint; NT_{90}, PsV NAb 90% neutralization endpoint; $NT_{partial}$, PsV NAb partial neutralization endpoint.

Table 3
HPV 16 and HPV 18 PsV NAb, cLIA and TIgG antibody levels by study group.

	n	7 months	*n*	18 months	*n*	24 months	*n*	36 months
HPV 16								
Group 1								
PsV NAb (NT_{100})	190	9897 (4915; 19,930)	99	898 (446; 3641)	190	1339 (446; 2441)	85	1339 (446; 1808)
PsV NAb (NT_{90})	161	19,930 (9897; 40,135)	84	1339 (1339; 4915)	169	2441 (665; 4915)	65	2441 (665; 2441)
PsV NAb ($NT_{partial}$)	177	80,822 (80,822; 80,822)	92	9897 (9897; 9897)	182	9897 (4915; 9897)	82	9897 (4915; 9897)
cLIA	251	8103 (3641; 16,318)	100	1480 (812; 3641)	200	1480 (735; 2697)	85	1480 (602; 2441)
TIgG	251	9897 (5432; 18,034)	100	1097 (735; 3641)	200	1097 (545; 2441)	85	1097 (446; 2441)
Group 2								
PsV NAb (NT_{100})	190	7332 (4915; 19,930)	94	1339 (665; 2441)	184	1339 (665; 2441)	85	1339 (665; 2441)
PsV NAb (NT_{90})	160	9897 (4915; 40,135)	80	2441 (665; 4915)	155	2441 (665; 4915)	70	2441 (665; 9897)
PsV NAb ($NT_{partial}$)	173	80,822 (40,135; 80,822)	84	9897 (9897; 14,765)	167	9897 (9897; 9897)	76	9897 (4915; 9897)
cLIA	254	7332 (3641; 13,360)	99	1998 (898; 3641)	187	1808 (898; 3294)	85	1636 (735; 2981)
TIgG	252	8955 (4915; 14,765)	98	1480 (812; 4447)	187	1212 (665; 3294)	85	1212 (602; 2981)
Group 3								
PsV NAb (NT_{100})	202	3641 (2441; 4915)	91	446 (330; 1339)	196	446 (330; 1339)	99	665 (221; 1339)
PsV NAb (NT_{90})	184	4915 (4915; 9897)	86	665 (446; 1808)	171	665 (446; 2441)	84	1339 (665; 2441)
PsV NAb ($NT_{partial}$)	201	40,135 (40,135; 80,822)	83	9897 (4915; 9897)	197	4915 (2441; 9897)	98	4915 (2441; 9897)
cLIA	276	3641 (1808; 7332)	96	812 (446; 1480)	210	812 (446; 1636)	99	735 (365; 1636)
TIgG	276	4447 (2208; 8103)	96	602 (365; 1212)	210	493 (299; 992)	99	545 (270; 992)
HPV 18								
Group 1								
PsV NAb (NT_{100})	192	2441 (1339; 4915)	90	330 (110; 665)	182	164 (81; 446)	72	164 (81; 330)
PsV NAb (NT_{90})	176	4915 (2441; 9897)	82	330 (164; 1339)	166	330 (164; 665)	70	330 (164; 665)
PsV NAb ($NT_{partial}$)	195	40,135 (19,930; 80,822)	99	4915 (2441; 9897)	196	2441 (1339; 9897)	85	2441 (665; 4915)
cLIA	251	1212 (735; 2441)	100	134 (81; 270)	200	148 (67; 330)	85	122 (55; 270)
TIgG	251	1480 (812; 2981)	100	181 (81; 330)	200	134 (60; 270)	85	134 (60; 299)
Group 2								
PsV NAb (NT_{100})	190	2441 (1339; 4915)	86	545 (221; 1339)	177	330 (164; 898)	81	330 (164; 665)
PsV NAb (NT_{90})	171	4915 (2441; 9897)	83	665 (330; 1808)	160	665 (330; 1339)	73	665 (221; 1339)
PsV NAb ($NT_{partial}$)	190	40,135 (19,930; 80,822)	93	9897 (2441; 9897)	180	4915 (2441; 9897)	84	2441 (1339; 7332)
cLIA	254	1808 (812; 2981)	99	221 (110; 545)	187	245 (122; 665)	85	200 (99; 545)
TIgG	251	1808 (992; 3294)	98	221 (122; 493)	187	181 (90; 403)	85	221 (90; 545)
Group 3								
PsV NAb (NT_{100})	206	1339 (665; 2441)	80	164 (81; 330)	176	164 (55; 330)	79	110 (81; 330)
PsV NAb (NT_{90})	198	2441 (1339; 4915)	86	240 (81; 665)	171	164 (81; 446)	70	164 (81; 665)
PsV NAb ($NT_{partial}$)	213	19,930 (9897; 40,135)	95	2441 (1339; 9897)	209	2441 (665; 4915)	102	898 (330; 2441)
cLIA	282	665 (299; 1480)	98	99 (37; 148)	215	110 (37; 221)	102	90 (33; 200)
TIgG	281	735 (365; 1480)	98	90 (40; 200)	215	74 (37; 181)	102	74 (30; 200)

Antibody levels are expressed as median geometric mean titre (Q1; Q3).
Abbreviations: PsV NAb, pseudovirus neutralizing antibody; cLIA, Merck competitive Luminex immunoassay; TIgG, Merck total IgG Luminex immunoassay; NT_{100}, PsV NAb 100% neutralization endpoint; NT_{90}, PsV NAb 90% neutralization endpoint; $NT_{partial}$, PsV NAb partial neutralization endpoint.

Table 4
HPV 16 and HPV 18 antibody levels at 36 months by baseline serologic result.

Baseline serologic result	Antibody level[a]					
	n	PsV NAb (NT_{100} GMT)	*n*	cLIA (mMU)	*n*	TIgG (mMU)
Anti-HPV 16						
Negative	271	665 (330; 1808)	270	1097 (493; 2441)	241	898 (403; 2208)
Positive	6	1339 (330; 1808)	6	1480 (735; 2441)	34	735 (403; 1212)
p-Value		0.670		0.770		0.129
Anti-HPV 18						
Negative	234	164 (81; 446)	274	134 (55; 330)	205	122 (55; 365)
Positive	3	221 (40; 665)	2	446 (270; 812)	70	148 (55; 299)
p-Value		0.911		0.145		0.970

Abbreviations: PsV NAb (NT_{100} GMT), pseudovirus neutralizing antibody geometric mean titre, 100% neutralization; cLIA (mMU), Merck competitive Luminex immunoassay, milli-Merck units; TIgG (mMU), Merck total IgG Luminex immunoassay, milli-Merck units.
[a] Antibody levels are expressed as median geometric mean titre (Q1; Q3)

Table 5
Group 3 HPV 16 and HPV 18 antibody levels at 36 months by baseline HPV DNA result.

Baseline HPV DNA result	Antibody level[a]					
	n	PsV NAb (NT_{100} GMT)	*n*	cLIA (mMU)	*n*	TIgG (mMU)
HPV 16						
Negative	97	665 (221; 1339)	96	735 (365; 1339)	96	493 (270; 992)
Positive	11	1339 (330; 1808)	11	1097 (365; 2441)	11	992 (493; 1808)
p-Value		0.127		0.235		0.090
HPV 18						
Negative	82	134 (81; 330)	105	81 (27; 200)	105	67 (30; 181)
Positive	2	270 (221; 330)	2	245 (245; 270)	2	365 (299; 403)
p-Value		0.278		0.140		0.074

Abbreviations: PsV NAb (NT_{100} GMT), pseudovirus neutralizing antibody geometric mean titre, 100% neutralization; cLIA (mMU), Merck competitive Luminex immunoassay, milli-Merck units; TIgG (mMU), Merck total IgG Luminex immunoassay, milli-Merck units.
[a] Antibody levels are expressed as median geometric mean titre (Q1; Q3).

unchanged through to 36 months. This is encouraging from a public health perspective and suggests that detectable antibodies may be maintained long-term following a 2-dose vaccine schedule in young girls.

Correlation coefficients for HPV 18 for all three assays were very similar, whereas for HPV 16, correlation between the PsV NAb and the TIgG assay was closer than either the PsV NAb or TIgG assays vs. the cLIA. There were a number of subjects with low levels of HPV 16 cLIA antibodies who displayed high levels of PsV NAb. For HPV 18, the cLIA and PsV NAb were more closely correlated. For those samples which lost detectable HPV 18 cLIA antibodies, the corresponding PsV NAb levels were typically low, confirming the close correlation. These findings likely reflect the more limited array of HPV antibodies detected by the cLIA due to its monoclonal antibody design or may reflect the composition of the PsV. Of interest, Hernandez et al. reported that HPV 16 antibodies detected by enzyme immunoassay (EIA) against either L1 or L1-L2 VLPs correlated well with the results of a PsV NAb assay. However, for HPV 18, EIA antibodies against L1-L2 VLPs correlated better with the PsV NAb assay than EIA antibodies against L1 VLPs. These authors suggest that L1-L2 VLPs likely more closely resemble native virions than L1 VLPs [17].

We demonstrated that Gardasil® induces higher HPV 16 antibody levels than HPV 18, regardless of the assay used to measure the responses. This is consistent with other reported cLIA responses to Gardasil® vaccine [4,5,18]. We previously reported that the HPV 16 and 18 PsV preparations used for the present study demonstrated similar reporter plasmid packaging efficiency [10], so this is unlikely to explain the observed differences. In addition, the measured PsV NAb titres could have been affected by the amount of L1 protein in the respective PsV preparations. The PsV L1 content has been shown to vary among HPV genotypes [19] and the HPV 16 PsV preparation in our study contained two to three times more L1 than the HPV 18 PsV. Of interest, the infectious unit titre of the HPV 16 PsV was approximately two times higher than that of HPV 18. These factors, as well as the packaging efficiency of the PsV, could have resulted in differences in the measured HPV 16 and 18 antibody levels. In contrast to Gardasil®, the Cervarix® vaccine induces similar antibody levels in women > 18 years of age for both HPV 16 and 18 [20] and antibody levels for both HPV 16 and 18 are higher than those induced by Gardasil®. The significance of the disparities in antibody titres induced by the two vaccines and their relevance to long-term persistence of vaccine-induced antibodies is unknown, given that very low levels of HPV antibodies have been shown to be protective in animal models [21].

We did not detect higher levels of antibodies at 36 months among subjects who were baseline HPV 16 or 18 seropositive, an observation similar to that of Ngan et al. with Cervarix® vaccine [22]. In contrast, Giuliano et al. [18] and Villa et al. [4] reported that baseline seropositive individuals demonstrated significantly higher anti-HPV responses following Gardasil® vaccine than those who were seronegative at baseline. We also were unable to demonstrate a significant difference in antibody responses at 36 months among subjects who were baseline HPV 16 or 18 DNA positive vs. negative, similar to the observations of Villa et al. [4]. Giuliano et al. [18] demonstrated that baseline HPV 16 and 18 DNA negative subjects had similar post-vaccine responses as baseline DNA positive subjects, except when subjects were both seropositive and DNA positive at baseline. Opalka et al. [3] reported that baseline HPV DNA positive subjects generally had higher titres at 48 months compared to subjects who were HPV DNA negative at day 0 or month 7. As our study had small numbers of baseline cLIA and PsV NAb seropositive and baseline DNA positive subjects, we lack the statistical power to assess potential differences in antibody responses for these subjects.

Given the high baseline HPV 16 and HPV 18 TIgG seropositivity among the study groups, it is unclear if all the detected TIgG antibodies are type-specific and/or neutralizing. It is unlikely that substantial numbers of 9–13-year-old girls would have sexually acquired HPV 16 and/or HPV 18 infections [23]. The majority of baseline TIgG seropositive subjects displayed antibody levels near the assay cut-off (data not shown), whereas post-vaccine levels were substantially higher in virtually all subjects. The low baseline seropositivity rates with both the cLIA and PsV NAb assays suggest that the high proportion of TIgG antibodies detected at baseline reflects low specificity of the TIgG assay, or cross-reactivity with other HPV types, such as those associated with cutaneous warts which are commonly acquired in childhood [24]. Safaeian et al. [25] observed a high HPV 16 baseline seropositive rate among 18–25-year-old women tested with the Glaxo-Smith-Kline HPV 16 EIA compared to the cLIA and a PsV NAb assay, and noted that agreement between the cLIA and the EIA was improved by raising the cut-off of the EIA. Brown et al. suggested that the high specificity of the cLIA may make it a more suitable assay for classifying baseline seropositivity, whereas the TIgG assay detects a broader array of HPV antibodies with high sensitivity and may be more suitable for serological follow-up of vaccinated subjects over time [15]. A modest upward adjustment of the TIgG assay cut-off would considerably reduce the number of individuals we identified as seropositive at baseline, but such an adjustment would require verification that the sensitivity of the assay for assessing post-vaccine responses would not be compromised.

We demonstrated that the PsV NAb assay sensitivity can be increased by determining partial neutralization endpoints. Both NT_{90} and $NT_{partial}$ endpoints consistently yield 2- to 8-fold higher GMTs than NT_{100}. While only 85–86% of subjects remained seropositive for HPV 18 at 36 months by both cLIA and PsV NAb (NT_{100} endpoint) assays, all subjects had detectable HPV 18 neutralizing

antibodies at the $NT_{partial}$ endpoint. Thus, we conclude that the PsV NAb assay is more sensitive than the cLIA for detection of anti-HPV 18.

The PsV NAb assay is labour-intensive and not suitable for large-scale analyses, but it can serve as a useful supplementary assay. While the determination of the PsV NAb endpoints may have a subjective component, we found that the assay is reproducible over multiple test batches and between operators (data not shown). Month 7 sera were initially tested together with baseline sera, and were later re-tested together with the 18-, 24- and 36-month sera. In nearly all cases, month 7 GMTs varied by no more than one dilution between test runs.

This study has some limitations. All PsV NAb assays for this report were performed with single lots of HPV 16 and 18 PsV. PsV NAb titres could be affected by variable inter-batch packaging efficiency of the RFP reporter plasmid but GMTs can be consistently derived by calibration of PsV batches using standard sera [26,27]. PsV NAb assays using RFP as the reporter gene are read by microscopy, whereas assays using secreted embryonic alkaline phosphatase are machine-read, and are potentially subject to lower variability. However, our initial validation studies and repeat testing of 7-month samples which had been earlier tested together with baseline samples revealed no more than 2-fold variation in GMTs between test runs and different technologists. Sequence variations between PsV prepared with the National Institutes of Health L1 plasmids and those used to construct the VLPs for the Merck cLIA and TIgG assays could also account for some variability between assays, as might the L2 component which is present in HPV 16 and 18 PsV, but not in the vaccine VLPs used in the Merck assays.

5. Conclusion

In summary, our study showed high correlation between HPV antibody levels measured by the PsV NAb and the Merck cLIA and TIgG assays. All three assays have similar sensitivity for detection of post-vaccine HPV 16 antibodies, but for HPV 18 both the PsV NAb and TIgG assays are more sensitive than the cLIA. The fact that three discernible GMT endpoints (NT_{100}, NT_{90} and $NT_{partial}$) were consistently derived by using a PsV NAb assay illustrates the challenges and complexities of defining immunoassay cut-offs for the assessment of HPV type-specific vaccine- and/or naturally induced antibodies. Unless assay cut-offs can be more accurately defined and the component elements better characterized, correlates of HPV seroprotection will remain elusive. A study is in progress to assess the 10-year durability of HPV antibody responses among subjects immunized with two vs. three doses of Gardasil®.

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