Multiplex immunoassay to measure antibody response to nine HPV vaccine types

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Abstract

Well-characterized HPV serology assays are required to evaluate performance of biosimilar candidate vaccines, reduced dosing schedules and novel administration methods. We report characterization of an expanded assay, M9ELISA, that detects antibodies to HPV virus-like particles (VLP) of nine types using direct IgG ELISA on the Meso Scale Discovery (MSD) electrochemiluminescence platform. The method is based on the previously published M4ELISA which detects antibodies to HPV6,11,16, and 18. It has been modified to add detection of antibodies to HPV31,33,45,52 and 58, and to streamline assay and reduce background.

The M9ELISA plates were prepared with purified type specific L1+L2 VLPs coated on 10-spot/well standard MSD microplates. Results of ELISA on three serial dilutions of serum were read on MSD imager, and titers calculated using the parallel line method. Evaluations included dynamic range, assay reproducibility, and stability over time. We compared M9ELISA results to those from a pseudovirion-based neutralization assay in sera from a mixed cohort of unvaccinated and vaccinated individuals (n = ~116) and to competitive Luminex immunoassay (cLIA) results in sera from a predominantly unvaccinated cohort (n=4426).

The linear range of the assay extended over 5 logs, with inter-assay reproducibility coefficient of variation   25% for all types. The pre-coated plates were stable for at least 2 years. Spearman correlation of antibody titers showed excellent correlation with PBNA (r=0.86–0.97) and moderate correlation (r = 0.52–0.68) with cLIA. Thus, the M9ELISA can serve as a useful platform for high-throughput, sensitive and simultaneous quantitation of the antibody responses to nine HPV vaccine types.

Keywords

HPV Antibodies; Serology; ELISA; Multiplex; Meso Scale Discovery

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1. Introduction

Vaccination with any of the three marketed human papillomavirus (HPV) vaccines, bivalent vaccine (2vHPV), quadrivalent HPV vaccine (4vHPV) and 9-valent HPV vaccine (9vHPV) has proven to be successful in eliciting a strong immune response, predominantly a long-lasting IgG response; the initial clinical trial participants continue to be protected from disease due to vaccine types (Arbyn and Xu, 2018; Kjaer et al., 2020a; Kjaer et al., 2020b; Porras et al., 2020). Because no serologic correlate of protection has been established, the approval of biosimilar vaccines, or evaluation of vaccination strategies such as further reduction in the vaccination series to a single-dose, or alternate delivery mechanisms to address vaccine cost or shortage, have relied on establishing immunoequivalency/non-inferiority to the current vaccines (Harper and DeMars, 2017). To do this reliably and reproducibly, validated serological assays are critical. We previously developed a reproducible, stable and sensitive multiplex assay to detect IgG response against virus-like particles of the 4vHPV types on the Meso Scale Discovery platform (M4ELISA)(Panicker et al., 2015). This platform allows multiplexing up to10 antigens within a well using a 10-spot plate, allowing space to expand on the previous M4ELISA which was coated on 7-spot plates. The assay requires less antigen input per spot for plate coating than traditional ELISAs and utilizes electrochemiluminiscent technology which allows for low sample volumes, a large dynamic range and quick read times (~70–90sec per plate). Though pseudovirion-based neutralization assay (PBNA) is well-known as the gold standard for HPV antibody testing, the assay is labor and time -intensive, low-throughput, and utilizes larger sample volumes to test nine types in singleplex format. Binding assays using conformationally intact VLPs have been shown to correlate well with neutralization assays, making the high-throughput binding assay a good surrogate when testing large sample numbers (Schiller and Lowy, 2009; Pinto et al., 2018). The current manuscript describes the assay performance characteristics of the M9ELISA for detection of antibodies to HPV6,11,16,18,31,33,45,52 and 58. The optimized assay is compared to PBNA as well as competitive Luminex immunoassay (cLIA) (Roberts et al., 2014), which was the assay used in 4vHPV and 9vHPV clinical trials.

2. Materials and Methods

2.1 Antigen preparation and plate coating

The antigen for each HPV was conformationally intact type-specific virus-like particles (VLPs) comprised of L1 and L2 proteins. VLPs for HPV6,11,16,18,31,33,45,52,58 were produced in Human Embryonic Kidney cells 293TT (HEK293TT) transfected with expression plasmids (Received from Dr. John Schiller, NCI, USA) and purified as described in Panicker et.al 2015 (Panicker et al., 2015) with minor modifications. Optiprep™ was removed by diafiltration with Dulbecco's-PBS (Thermo Fisher, Waltham, MA) + 0.5M sodium chloride using Amicon Spin columns (Millipore Sigma, Burlington, MA). The absorbance reading at 320 nm for each VLP fraction was required to be < 0.05 (Nanodrop 2000 spectrophotometer) to verify that Optiprep ™ was removed. Quality measures for the VLPs included protein concentration, transmission electron microscopy (TEM), and ELISA reactivity to conformation and type-specific monoclonal antibodies greater than or equal to

the laboratory reference VLP stock (Labnet, 2009). Frozen quality-verified fractions were thawed and pooled to create VLP preps that were re-qualified and stored at −80°C.

Qualified VLP preps were shipped overnight on dry ice to Meso Scale Discovery (MSD, Rockville, MD) for 10-spot multi-array printing in 96-well format. MSD thawed VLPs just prior to coating plates and diluted each prep to 80 μg/ml in PBS+ BSA buffer for printing using optimized parameters as described (Panicker et al., 2015). Printed plates were shipped on cold packs to CDC and stored at 4°C until use. Each plate printing at MSD is considered a separate plate lot. Different plate lots may be printed with the same or different VLP preps. Typically, the interval between lot printing is 9 to 12 months. Quality control checks to evaluate VLP integrity and spot specificity after printing were performed using optimized dilutions of type-specific monoclonal antibodies HPV6.M48, HPV11.H3, HPV16.V5, HPV18.J4,HPV31.A6, HPV33.J3, HPV45.N5, HPV52.D11, HPV58.J3.6 (Gift from Dr. Neil Christensen, Pennsylvania State University, USA) as well as comparison of antibody titers generated with a panel of sera with previously known reactivity to 9vHPV vaccine types using assay procedure described below.

2.2 Samples and controls

The HPV16 International Standard (IS, 05–134) and HPV18 IS (10–140) were obtained from National Institute for Biological Standards and Controls [NIBSC], Potter's Bar, UK. Reference and control samples were prepared as described below from residual heat-inactivated sera from individuals vaccinated with 4vHPV or 9vHPV (gift of Merck & Co, Inc.) as well as anonymized residual sera from previous epidemiologic studies of vaccinated and unvaccinated individuals.

Three 9vHPV vaccinee sera were pooled and diluted in antibody depleted human serum (Valley Biomedical, Winchester, VA) to form the reference used on every assay plate. This reference sample was calibrated to the HPV16 and HPV18 IS and used to determine international units (IU)/ml for HPV16 and 18 and arbitrary units/ml (AU/ml) for other types.

Medium and low positive controls were included on every test plate. These included sera from a vaccinated individual diluted in antibody depleted human serum and a pool of sera from 11 individuals collected during the pre-vaccine era. To provide a consistent negative result for all types, sera from one naïve individual was diluted 1:4 in antibody depleted human sera and used as negative control on every plate.

To establish assay parameters of stability and reproducibility we prepared a panel of residual sera from vaccinated and unvaccinated individuals inclusive of positive controls (n=17) for repeated testing. Residual sera (n=4426) from predominantly unvaccinated U.S. individuals collected between 2005–2006 that had been previously tested in 9-valent cLIA (Pharmaceutical Product Development LLC (PPD), Wilmington, NC) were retested with M9ELISA to compare assay results. A set of residual sera from a mixed cohort of unvaccinated and vaccinated individuals (n=116–138) tested with M9ELISA were also tested with the nanoluciferase pseudovirion-based neutralization assay (nlucPBNA).

2.3 HPV 9-plex VLP based IgG ELISA (M9ELISA)

Serial 3.16-fold dilutions of human serum samples started at 1:100 were prepared in assay diluent [0.1X Diluent 100™ (MSD, Rockville, MD) in Phosphate-buffered saline (pH 7.4), 0.1% Tween 20, (PBST)]. A minimum of 3 dilutions were tested for each sample. Serial dilution was performed by Janus® automated liquid handling workstation (PerkinElmer, Waltham, MA). All other steps were performed manually. Mouse anti-human IgG (Fc specific) (Biotrend Chemicals LLC, Destin, FL) was sent to MSD for SULFO-TAG™ labeling and stored in individual use aliquots at 4°C. The labeled secondary antibody was diluted at 1 μg/ml in assay diluent immediately before use.

Plates stored at 4°C were allowed to acclimate to room temperature (24°C± 2) before testing. Plates were blocked for 1 h with 5% ECL™ Blocking Agent in 1X PBST at room temperature, 150 μl per well at 650rpm. After removal of the blocking agent, each subsequent addition involved incubation (37°C for 1 h with shaking at 650 rpm) followed by four washes with 150 μl per well of 1X PBST using an automated plate washer (ELx405VRS, Biotek, Winooski, VT). The first addition was 50 μl of sample per well and the second was 25 μl of SULFO-TAG™ labeled secondary antibody. After the last wash, 150 μl of 1X Read Buffer T (MSD) was added to each well and the plate was immediately read on the Sector Imager 6000 (MSD) or Quickplex SQ120 (MSD). Raw signal for each spot in relative light units (RLU) was exported to Microsoft Excel for use in subsequent calculations of dynamic range and antibody titers (without subtraction of BSA signal). We used the parallel line method (PLL) as described in the WHO HPV Labnet Manual (Grabowska et al., 2002; Labnet, 2009) to calculate antibody titers relative to the reference. The same basic approach was used for evaluation of plates with type-specific mouse monoclonal antibodies except that only one dilution was used and the secondary antibody was goat anti-mouse SULFO-TAG™ labeled IgG (MSD).

In addition to changing from 7-spot to 10-spot plates to allow inclusion of additional antigens, changes from the M4ELISA method included the assay diluent, sample volume per well and secondary antibody. We changed the assay diluent from 1% ECL™ Blocking Agent (GE Healthcare Biosciences, Piscataway, NJ) to 0.1X Diluent 100™ (MSD) in PBST to address background. The volume of diluted sample per well was changed from 25 μl to 50 μl to improve consistency of results (reduced spot-drop off). The secondary antibody was labeled with SULFO-TAG™ for direct detection thereby reducing the incubation and washing steps required with indirect detection.

2.4 Nanoluciferase Pseudovirion- based Neutralization Assay (nluc-PBNA)

Pseudovirions for each of the 9vHPV types were produced and assay performed as described by Pastrana et.al.(Pastrana et al., 2004) with the following modifications. The reporter plasmid coding for secreted nanoluciferase (Geoghegan et al., 2017)(gift from Dr. Christopher Buck, NCI, USA) was substituted for secreted alkaline phosphatase and the maturation phase of the pseudovirions was 48 instead of 24 hours. Post-vaccination serum with known neutralization titer for 9vHPV types and type-specific monoclonal antibodies were used as positive controls. Negative controls were neutralization buffer only (no serum) and antibody depleted human serum. Secreted nanoluciferase activity in the samples was

measured using Nano-Glo® Luciferase Assay kit (Promega, Madison,WI) and results read on Synergy H1 (Biotek, Winooski, VT). The antibody titer showing 50% neutralization activity was calculated using the 4-parameter curve fit in Prism ver 6.07 or 7.05 (GraphPad, La Jolla, CA). Serum samples were considered positive if HPV type specific neutralization titers were   100. Assay cut-off of 100 was established based on the median reactivity observed with BPV pseudovirus in a subset of vaccinated and unvaccinated serum.

2.5   Establishing assay performance characteristics

2.5.1   Dynamic Range and Limits of Detection—The reference sera were serially diluted 3.16-fold from 1:10 to $1:3.14\times10^6$ and tested in duplicate along with assay diluent over three days on two different lots of printed plates to determine the assay's dynamic range. The minimum detection signal was set at 350 for each VLP spot based on average + 3SD of the signal from assay diluent. The maximum signal was set at 1×10^6 RLU, the manufacturer's recommended upper limit for reproducible signal.

To calculate limits of detection and quantitation for antibody titers, reference sample was serially diluted 2-fold starting at neat to 1:8192 dilution. The expected type-specific titers for each dilution was assigned based on known results for the reference sample. Each of the dilutions was handled as a separate sample for type-specific determination of titers in M9ELISA with PLL calculation. Blank wells containing only assay diluent were included. The samples were tested in duplicate by two operators each on two plate lots on three separate days. The type-specific titers for each sample on all runs were used to calculate the lower limit of quantitation (LLOQ), with the degree of imprecision and bias at LLOQ   30% and with the total error (imprecision + bias) at LLOQ   40% (FDA-2013-D-1020, 2018).

2.5.2   Assay Reproducibility—Results of M9ELISA on the panel of 17 samples (described in section 2.2) was used to determine assay reproducibility. Intra-lot coefficient of variation was determined by testing the panel on three plates within a lot on three non-consecutive days for each plate lot. This was repeated for two more plate lots. Inter-assay coefficient of variation across three plate lots was also determined with testing performed on one plate per lot on three non-consecutive days. For all experiments, samples were tested once on each plate. Antibody titers below limit of quantitation were removed from calculations prior to determining median % coefficient of variation (CV).

2.5.3   Plate Stability—The panel was tested on three plate lots over a 12-month period and for 22-months for two plate lots. Time as shown in months was based on the date of receipt in the laboratory not the date of plate printing. Median fold change in antibody levels was calculated at each month compared to the first run.

2.5.4   Pre-diluted Sample Stability—Eight serum samples of varying antibody titer were diluted in preparation for M9ELISA but in volumes permitting multiple assays. The dilutions were aliquoted and one set was tested on the day of dilution. The remaining aliquot sets were stored at 4°C or −20°C for varying time periods. Dilutions stored at both temperatures were tested after three days, one week and three weeks. The −20°C aliquots were also tested after two months following one and two freeze-thaw cycles. Ratio of

fold-change in signal as well as %CV of the antibody titers at various time-points were calculated.

2.6 Assay comparisons with nlucPBNA and HPV-9 cLIA

Spearman's correlation was calculated for all samples tested for a given type on M9ELISA and nlucPBNA. For comparison to cLIA, M9ELISA results from 4426 serum samples tested with 1%ECL™ Blocking Agent as diluent but titers calculated without background subtraction were used. All samples below the quantitation limit for cLIA and M9ELISA were assigned a value half the limit of quantitation for each type for cLIA based on Roberts et.al (Roberts et al., 2014), and for M9ELISA based on results in this manuscript. M9ELISA assay antibody levels (expressed as IU/ml or AU/ml) were compared with cLIA results (mMU/ml or IU/ml). For HPV16, 1 IU/ml =~11.8 mMU/ml (Personal Communication, PPD) and for HPV18, 1 IU/ml =~5.26 mMU/ml (Brown et al., 2014). Spearman's correlation was calculated between assays for all samples using Prism ver 6.07 or 7.05 (GraphPad).

3. Results

3.1 Assay performance characteristics

M9ELISA had a broad dynamic range with assay linearity extending to 5–6 log range for all types as shown in Fig. 1 and Supplemental Fig.1. The lower limit of quantitation for HPV6,11,31,33,45,52,58 were 0.2,0.4,1.6,3.0,2.3,1.6,2.2 AU/ml respectively, and 1.0 and 0.3 IU/ml for HPV16 and 18 (Supplemental Fig.2).

Specificity of binding to different VLP spots within the same well was confirmed using type-specific monoclonal antibodies. Depending on the HPV type, a 400–1000-fold signal above background was observed with type-specific mAbs, and <10-fold above background for the non-specific types (Supplemental Table 1).

Assay precision within plate lot for the three plate lots tested ranged from 4.9 through 17.3% (Fig. 2a). Assay variation between plate lots ranged from 12.04 through 21.58% (Fig. 2b), slightly higher than the intra-lot variation. While different plate lots may use the same VLP preps, the comparisons reported are for plate lots that also differed in VLP preps.

The median fold change in antibody titers evaluated over 2 years was minimal for each of the 9vHPV types printed (Supplemental Fig.3). To further increase testing throughput, the stability of pre-diluted samples stored at 4°C and −20°C were also evaluated. Median-fold change in raw signal ranged from 0.7–1.1-fold at various time points, with diluted sample stored at 4°C for 3 weeks showing the greatest fold change. At the latest time point of 2 months at −20°C, even after 2 freeze-thaw cycles, the fold change was minimal (0.9–1) at all dilutions of the samples tested. The average % CV in antibody titers across types at this timepoint after 2 freeze-thaw cycles for HPV6,11,16,18,31,33,45,52,58 was 3.9%, 2.3%, 12.8%, 10.4%, 11.5%, 13.7%,16.1%, 13.3%, 12.5% respectively.

3.2 Assay Comparison with PBNA and cLIA

Antibody titers generated by M9ELISA were compared to PBNA and cLIA. Antibody titers correlated well across types when compared to PBNA (r >0.92, p<0.01) for all types except HPV45 which had lower correlation of r=0.86 (Fig. 3). Among the largely unvaccinated cohort, comparison of M9ELISA to cLIA yielded a moderate correlation across types with HPV18 having the lowest (r= 0.515, p<0.0001) and HPV6 the highest correlation (r= 0.683, p<0.0001) (Fig. 4).

4. Discussion

We describe the evaluation of a multiplex high-throughput ELISA that can reliably measure IgG responses to 9vHPV types. The assay uses methods that are an extension of the previously published M4ELISA which detected antibodies to 4vHPV types (Panicker et al., 2015). The M9ELISA is a robust assay with a large dynamic range and good precision as evidenced by coefficient of variation < 25% for each type. Reproducible measurement at low levels of quantitation makes it suitable to measure antibodies to 9 HPV types occurring after natural infection and/or HPV vaccination. Recently, Tsang et. al evaluated the reproducibility of several serological assays including the M9ELISA that have been or will be used to measure vaccine response (Tsang et al., 2020). The study consisted of 530 samples, from participants receiving varying doses of 2vHPVor 4vHPV up to 36-months post-vaccination, that were divided into multiple blinded aliquots to allow samples to be tested in duplicate by each assay. Data reported for M9ELISA HPV16 and 18 antibody titers showed good reproducibility, including among two-dose and single dose recipients (<10% CV and 0.99 intraclass correlation coefficient irrespective of group).

We compared antibody titers established with the M9ELISA to cLIA, which was the assay used in 9vHPV clinical trials (Roberts et al., 2014). The two assay formats differ with the cLIA recognizing antibodies of all classes to only one neutralizing epitope whereas M9ELISA detects all binding IgGs. Despite the differences, the correlation between antibody titers was moderate for all types ranging from 0.515 for HPV18 to 0.683 for HPV6 in the largely unvaccinated cohort. The level of correlation was similar to past studies evaluating 4vHPV types and was expected due to the generally low levels of antibodies elicited by natural infection (Wentzensen et al., 2011; Lin et al., 2013; Scherpenisse et al., 2013). Other than our study, correlation between ELISA and cLIA antibody titers have not been reported for additional 5 types targeted by 9vHPV. We also compared M9ELISA with PBNA, considered a gold standard for its ability to measure biological activity of the antibodies. The correlation was greater than 0.92 for all types except for HPV45 (r=0.86) using sera from a mixed cohort of unvaccinated and vaccinated individuals. Because we did not have the epidemiological data linked to these samples, we could not parse the groups into unvaccinated and vaccinated cohorts for a better comparison between M9ELISA and PBNA. However, previously published data on M9ELISA for HPV16 and 18 to secreted alkaline phosphatase PBNA showed moderate to good correlation of titers ranging from 0.75 and 0.91 for HPV16, 0.97 and 0.95 for HPV18 in single and full dose 2vHPVor 4vHPV recipients, respectively (Tsang et al., 2020). The better correlation between the M9ELISA

and PBNA vs M9ELISA and cLIA in this report is likely due to the different samples used for the comparisons in this report.

We continue to report titers in IU/ml for HPV16 and 18 to enable comparison between various serological assays in the field utilizing the same units. Collaborative efforts are underway to establish primary standards for each of the other 9vHPV types (Pinto et al., 2018). Once available, we will be able to re-titer our reference sample and establish an appropriate factor to convert past sample results in AU/ml to IU/ml for the remaining 7 types.

In summary, the M9ELISA is a high-throughput assay to detect IgG antibodies to 9vHPV types. Using good quality VLPs as antigen, the pre-printed plates are stable for over a year. The ability to pre-print large lots, ease of use and throughput make it particularly valuable assay for testing in large-scale studies. Although binding assays are not directly comparable to cLIA or PBNA, the M9ELISA has shown good correlation with results from those assays. In this study we do not report cut-offs for positivity by M9ELISA. There are challenges in setting cut-off values that are comparable across all HPV assays, due to lack of standardized serology reagents and difficulty obtaining sera from persons without HPV exposure. We are currently re-evaluating our methodology to establish cut-off values for seropositivity using children's sera and working collaboratively with other laboratories conducting HPV serology assays to determine a common approach.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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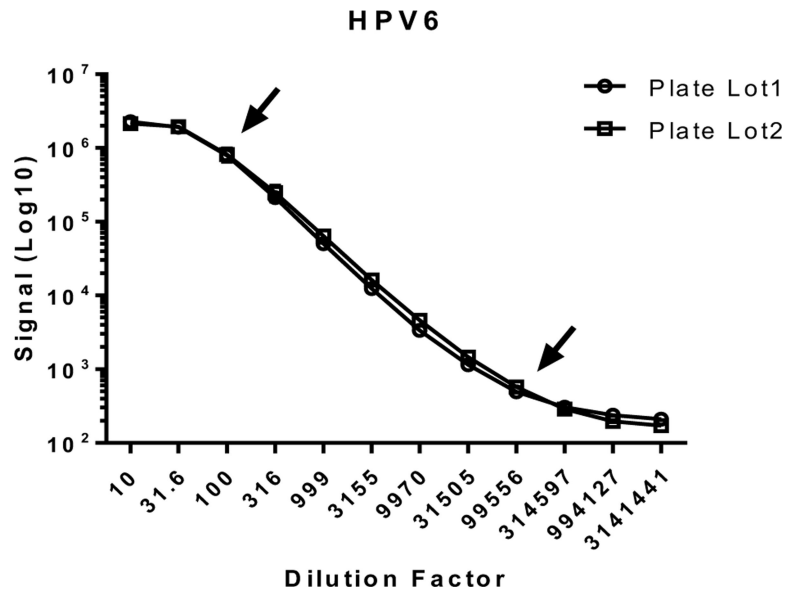
Fig.1.

Serial dilution of pooled post-vaccine sera tested in duplicate on two plate lots on three separate days. Representative figure with arrows highlighting the linear range and standard error between runs.
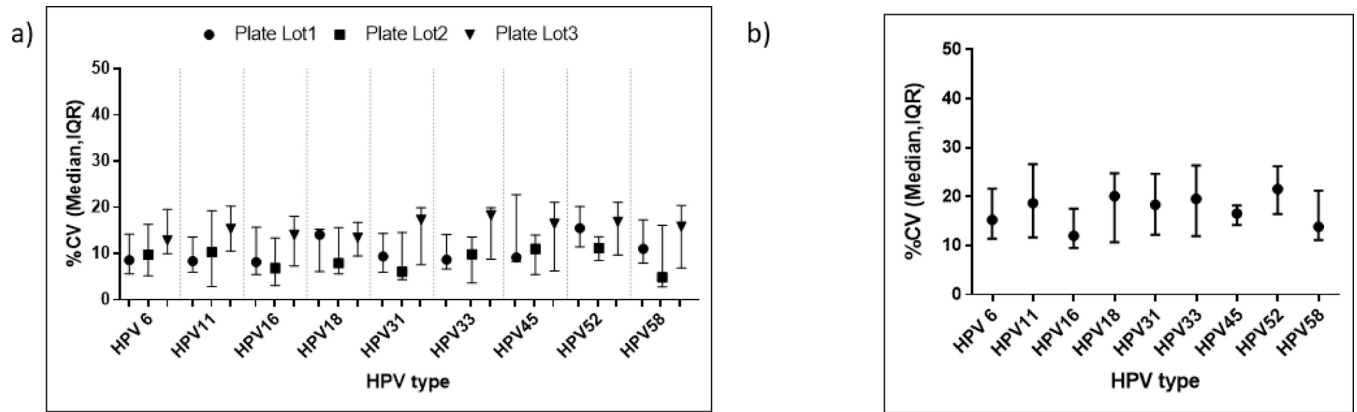
Fig. 2.
Inter-assay coefficient of variation (CV) across 3 plate lots a) Intra-lot CV (three plates within a plate lot on three non-consecutive days) b) Inter-lot CV (one plate per lot on three non-consecutive days)
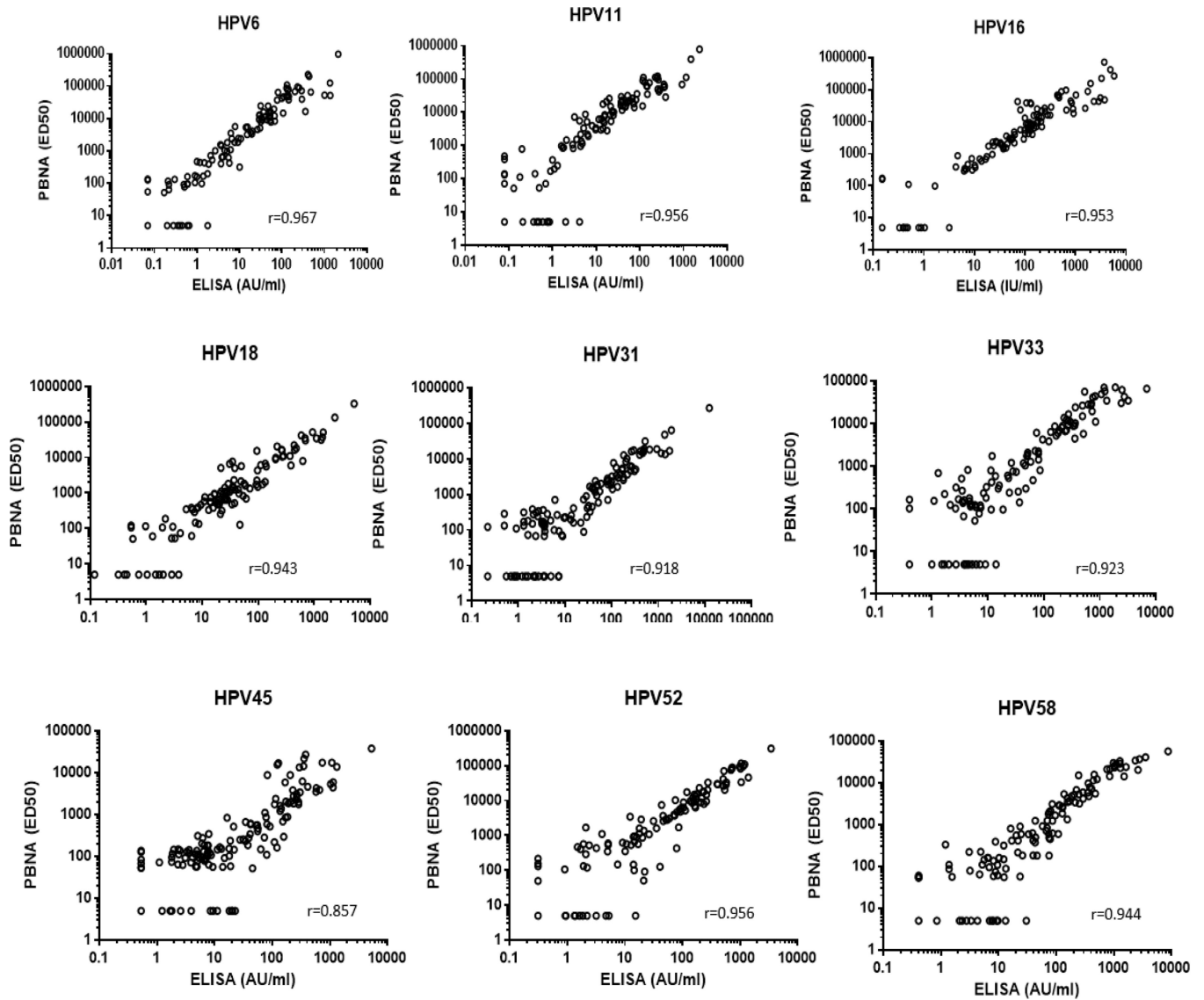
Fig. 3.
Correlation between antibody titers by M9ELISA and nluc PBNA among mixed sample set of unvaccinated and vaccinated samples (Spearman correlation, p-value <0.01)
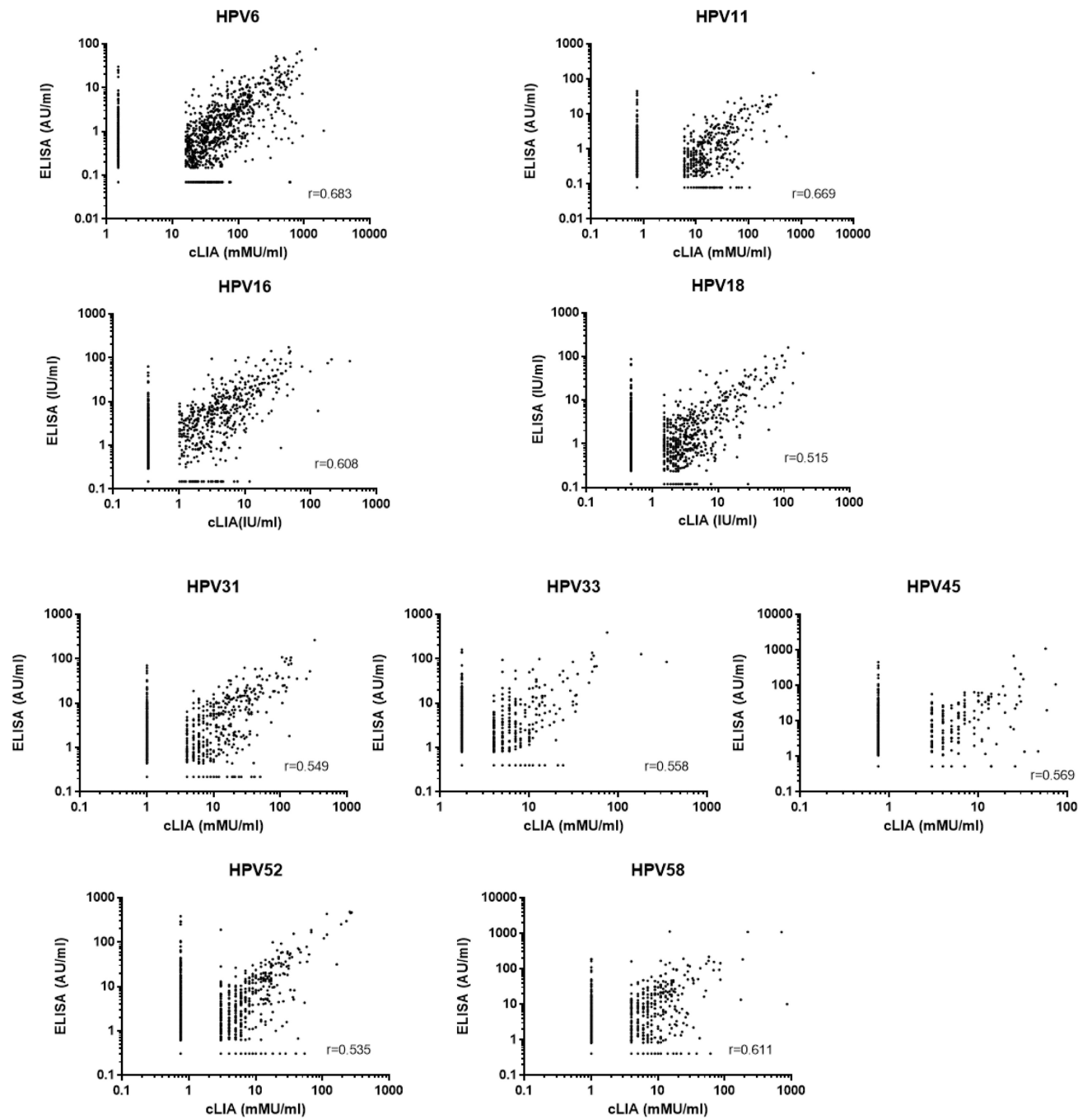
Fig. 4.
Correlation between antibody titers by M9ELISA and cLIA among unvaccinated samples
(Spearman correlation, p-value <0.000)