HHS Public Access

Author manuscript

J Immunol Methods. Author manuscript; available in PMC 2019 May 15.

Published in final edited form as:

J Immunol Methods. 2017 August ; 447: 31–36. doi:10.1016/j.jim.2017.04.004.

Description of a novel multiplex avidity assay for evaluating HPV antibodies☆

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Abstract

Limited data exists regarding antibody avidity for human papillomavirus (HPV). We describe development of a multiplex electrochemiluminescent avidity ELISA for four HPV types (HPV 6, 11, 16, 18) by adding a dissociating step to our established multiplex HPV VLP ELISA. Initial experiments exploring ammonium thiocyanate, sodium thiocyanate and guanidine hydrochloride (GuHCl) as dissociating agents identified GuHCl as most promising. Dissociation conditions with GuHCl were varied (concentration, incubation time, temperature) to select conditions with minimal impact on VLP integrity as measured with monoclonal antibodies to conformational epitopes. Avidity index (AI) was calculated based on a standard curve as ratio of bound IgG in GuHCl treated versus untreated sample. To evaluate our assay we determined AI in sera with known HPV titers. We selected 32 residual anonymized sera from individuals with a wide range of titers for HPV6, 11, 16, and 18. AIs were similar across multiple dilutions of serum within the assay's dynamic range and were reproducible with two plate lots. This assay will aid in understanding HPV antibody avidity and maturation in response to natural infection and varying vaccine schedules. This is the first report of a VLP-based multiplexed avidity ELISA that evaluates assay parameters for all nine HPV vaccine types.

Keywords

HPV antibodies; Avidity; Multiplex; Electrochemiluminescence; Human papillomavirus

1. Introduction

Production of antibody in response to infection or vaccination is fundamental to combating and preventing infectious disease. Antibodies bind their respective antigens through a myriad of non-covalent interactions. Repeated exposure to a specific antigen refines these interactions through affinity maturation, allowing antibodies to bind more tightly (Victora and Nussenzweig, 2012). The interaction of a single antibody to a single ligand is referred to as antibody affinity (or "intrinsic affinity"); while, antibody avidity (or "functional affinity")

☆The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the funding agency.

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is the interaction of a polyclonal population of antibodies to a complex ligand (Hedman et al., 1993). Because antibodies produced in response to infection or vaccination are heterologous, measuring antibody avidity is most relevant to understanding the functional efficiency of the humoral response. Antibody avidity has been correlated with the killing and neutralization of several human pathogens, with lower concentrations of high avidity antibody being needed for the opsonophagocytic killing of pneumococcus (Anttila et al., 1998; Anttila et al., 1999) and *Haemophilus influenzae* B (Schlesinger and Granoff, 1992; Granoff and Lucas, 1995), as well as the neutralization of respiratory syncytial virus (RSV) (Delgado et al., 2009) and others (Roost et al., 1995).

Antibody avidity can be assessed using several different methods (Hedman et al., 1993); but is commonly measured by modifying a traditional ELISA. This method employs the use of a chaotropic agent which disrupts the interaction of antibodies bound to their cognate antigen. The premise is that low avidity antibodies will dissociate and be washed away, allowing only high avidity antibodies to remain associated. Quantitatively, avidity can be expressed in several ways depending on the assay set up (Hedman et al., 1993). The ratio of the concentration of antibodies bound with and without treatment, i.e. avidity index (AI), is one of the more common ways to define avidity (Anttila et al., 1998; Licciardi et al., 2012; Almanzar et al., 2013; Scherpenisse et al., 2013; Boxus et al., 2014).

Though much is known about type and level of antibody response generated by HPV vaccination, there is no minimum level of correlate of protection against HPV infection (Castle and Maza, 2016). Antibody avidity could add information to titer results to provide better understanding of the changes in antibody response with alternate dosing schedules, number of vaccine doses, long-term response levels as well as differential response in special populations. Some studies have looked at antibody avidity for HPV (Kemp et al., 2012; Scherpenisse et al., 2013; Boxus et al., 2014; Einstein et al., 2014; Sankaranarayanan et al., 2016); however, they are mostly limited to HPV16 or 18. With the exception of a Luminex-based multiplex avidity assay (Scherpenisse et al., 2013), the current HPV avidity assays are singleplex colorimetric VLP-based ELISAs (Dauner et al., 2012; Boxus et al., 2014). A multiplex avidity assay would be beneficial, particularly in light of the recommendation for use of the 9-valent HPV vaccine in routine vaccination (Petrosky et al., 2015). This paper describes the method development and evaluation of a multiplex VLP-based IgG avidity ELISA using electrochemiluminescent detection for high-throughput, sensitive and type-specific testing of samples. This avidity assay for IgG antibodies to HPV6, 11, 16 and 18 is based on modification of our previously published M4ELISA (Panicker et al., 2015), but could easily be expanded to include the five additional vaccine types HPV 31, 33, 45, 52, and 58.

2. Materials and methods

2.1. Reagents and samples

HPV L1/L2 VLPs were produced in the HEK293TT cell line and purified by centrifugation using an Optiprep gradient as previously described (Buck and Thompson, 2007). Plasmid constructs used for generation of VLPs were a gift from J. Schiller (NCI, Bethesda, MD). Optiprep was removed from VLP preparation by diafiltration with Dulbecco's phosphate

buffered saline (DPBS) + 0.5 M NaCl using 50 kDa Amicon Filter (EMD Millipore, Billerica, MA). Quality of VLPs was assessed using type-specific monoclonal antibodies (gift from Dr. N. Christensen, Pennsylvania State University, USA) which bind conformational epitopes (Dillner and Unger, 2009) and compared to laboratory reference VLP pools (Panicker et al., 2015). VLPs were also viewed with transmission electron microscopy (TEM) to assess uniformity of shape and size. Following quality control checks, frozen VLP aliquots were sent on dry-ice to Meso Scale Discovery (MSD, Rockville, MD). MSD stored the VLPs at −80 °C prior to printing. VLPs (types 6, 11, 16, and 18) were coated at a concentration of 80 μg/mL for printing of 7-spot MSD plates for M4ELISA, as described previously (Panicker et al., 2015). A similar protocol was used to print 10-spot plates with HPV VLPs for types 6, 11, 16, 18, 31, 33, 45, 52 and 58 (M9ELISA). Plates were shipped and stored at 4 °C until used.

Guanidine hydrochloride (GuHCl, >99% purity) was purchased from Sigma (St Louis, MO) and reconstituted at various concentrations in 1× PBST-BSA [phosphate buffered saline with 0.1% Tween 20 (PBST) containing 1 mg/mL bovine serum albumin (BSA, Sigma Aldrich)]. Ammonium thiocyanate (NH_4SCN) and sodium thiocyanate (NaSCN) were purchased from Sigma and reconstituted at various concentrations in 1× PBST.

Type-specific monoclonal antibodies (mAb), H6.M48 for HPV6, H11.H3 for HPV11, H16.V5 for HPV16, H18.J4 for HPV18, H31.A6 for HPV31, H33.J3 for HPV33, H45.N5 for HPV45, H52.D11 for HPV52, and H58.J6.3 for HPV58, used for determining the effects of GuHCl on the conformation of VLPs, were received as a gift from Dr. N. Christensen (described in (Christensen et al., 1994; Christensen et al., 1996a; Christensen et al., 1996b; Rizk et al., 2008)).

Thirty de-identified residual serum samples from a population of naturally infected and quadrivalent HPV vaccinated persons were selected to evaluate the multiplex avidity assay. These samples were selected for their broad range in titer in previous M4ELISA and positivity for HPV 6, 11, 16, and 18; however, specific status of vaccination for individual samples is not known. Antibody titers ranged from 3.63–1134.13 arbitrary units (AU)/mL for HPV6, 0.66–1587.79 AU/mL for HPV11, 1.62–5665.4 International Units (IU)/mL for HPV16, and 0.9–3411.8 IU/mL for HPV18. A heat-inactivated pooled serum sample with high titer antibodies to HPV 6, 11, 16, and 18 (a gift from Merck, Inc.) was used as reference. A single known dilution of the reference sample was also used as an avidity control in the assay.

2.2. Selection of dissociation conditions for M4-AvELISA or M9-AvELISA

We conducted a preliminary evaluation of NH_4SCN, NaSCN, and GuHCl as denaturants and chose to explore treatment conditions with GuHCl that would introduce a dissociating environment while retaining VLP conformational epitopes. Effects of GuHCl concentration, incubation time and temperature on VLP conformational epitopes were evaluated. Briefly, multiplex ELISA plates were blocked using 5% ECL blocking reagent (GE Healthcare, Atlanta, GA) and washed as described for M4ELISA (Panicker et al., 2015). GuHCl in PBST-BSA, at concentrations ranging from 0 M (untreated) to 4 M, was added to wells and incubated (15 or 30 min) at either room temperature (24 °C ± 2) or 37 °C. Each treatment

condition was tested in quadruplicate per run. Incubated plates were washed four times with 1 × PBST and then 25 μL of pooled type-specific mAbs diluted in 1% ECL blocking reagent (concentration optimized to yield reproducible signal within detectable range for each type) was added to the plate and incubated for 1 h at 37 °C with shaking at 650 rpm. Plates were then washed four times with PBST and incubated with 25 μL of 0.5 μg/mL anti-Mouse IgG-SULFO-TAG® antibody (MSD, Rockville, MD) for 1 h at 37 °C with shaking at 650 rpm. Following incubation, plates were washed four times with PBST, and then 150 μl of 1 × Read buffer T (MSD) was added to each well. The plate was read on the Sector Imager 6000 (MSD). The effect of GuHCl on VLP integrity was determined by calculating the percent of mAb bound to treated VLPs compared to untreated VLPs (% of mAb bound = [Relative Light Units (RLUs) of treated well / RLUs of untreated well] * 100).

Conditions of 0–4 M GuHCl in PBST-BSA for 15 min at 37 °C were replicated on 3 different plate lots for the M4-AvELISA and 2 plate lots for the M9-AvELISA.

2.3. Multiplex avidity IgG ELISA (M4-AvELISA)

The avidity ELISA (M4-AvELISA) is a modification of the previously developed multiplexed ELISA (Panicker et al., 2015). The M4-AvELISA tests sera in duplicate rather than as a dilution series used in M4ELISA, which equated to four wells for each sample—two wells for “treated” and two wells for “untreated”. The serum dilution was selected to yield RLUs within the detectable range of the assay (between 1000 and 1,000,000 RLUs) for all types. If one dilution did not suffice for all types, then two dilutions were chosen and the avidity index was calculated for each type based on the lowest dilution that yielded RLUs within the detectable range. If the RLUs of the treated well were outside of the linear range when sample was tested at 1:100 dilution, avidity indices were not calculated for the given type. The reference sera serially diluted 3.16 fold from 1:31.6 to 1:1,000,000 in 1% electrochemiluminescent (ECL) blocking reagent (GE Healthcare Life Sciences, Pittsburg, PA) was used to generate a standard curve. An additional two wells per plate were used for duplicates of the avidity control (1:1000 dilution of the reference serum). The procedure followed the M4ELISA assay through the washes after the antibody incubation step (see Fig. 1 for assay flow chart). Rather than continuing to detection, 25 μL of 1 M GuHCl in PBST-BSA was added to two wells (“treated”) and PBST-BSA was added to remaining two wells (“untreated”). For the standard curve wells, 25 μL of PBST-BSA was added to each well. 25 μL of 1 M GuHCl in PBST-BSA was added to the two avidity control wells. The plate was incubated for 15 min at 37 °C with shaking at 650 rpm, and then washed. Detection and reading on the Sector Imager 6000 (MSD) followed M4ELISA protocol. Data from Discovery Workbench Software (MSD) was exported into Excel. If the RLUs of the duplicates (either treated or untreated) had a coefficient of variation (%CV) >20%, samples were considered failed for a particular type and subsequently retested for a final result. Avidity index was calculated as described below.

2.4. Calculating avidity index (AI)

A standard curve was used to determine the concentration of IgG Ab bound with and without dissociation treatment and the avidity index (AI) calculated as a ratio of those

concentrations. Thus, AIs closer to 1 correlate to high avidity, while AIs closer to 0 correlate to low avidity.

Arbitrary IgG antibody concentrations for each HPV type (designated as standard units per milliliter, SU/mL) were set for each dilution of reference serum. The standard curve for each HPV type was generated by plotting the natural log of SU/mL against RLUs. The plot was fitted with a 5-paramenter non-linear fit weighted by $1/y^2$ using GraphPad Prism version 5 (GraphPad, La Jolla, CA). The SU/mL for each sample was interpolated based on the standard curve for the respective HPV type. The AI was calculated as a ratio of the SU/mL of the treated versus the untreated sample. The AI was calculated for each duplicate and then averaged. If the AI %CV for the sample duplicate was >20%, the sample was considered failed for that type and retested.

2.5. Determination of dilution effect on AI

Six de-identified residual serum samples, known to be positive for antibodies to HPV 6, 11, 16, and 18 by M4ELISA were tested at several 3.16 fold dilutions that yielded signal within the range of the assay. The AI at each dilution was compared to the remaining dilutions tested to determine if dilution impacted AI.

2.6. Statistics

All statistical analyses were performed using GraphPad Prism version 5 or JMP version 10 (SAS, Cary, NC). Intra-assay variation for each HPV type on M4-AvELISA was calculated as the median coefficient of variation of samples run on two different days. Inter-assay precision was calculated as the median %CV of samples tested on three different days. Spearman correlation was used to determine correlation between antibody titer and AI.

3. Results

3.1. Effect of guanidine hydrochloride on VLP integrity for 9 HPV types

As shown in Fig. 2A–D, neither temperature (room temperature versus 37 °C) nor incubation time with GuHCl (15 min versus 30 min) significantly affected conformation of any VLP type as reflected in binding of mAb. However, GuHCl concentration did impact VLP conformation, with differences noted by type. The effect of GuHCl concentration was less dramatic for HPV 6 and 11 (Fig. 2A–B) than for HPV 16 and 18 (Fig. 2C–D) but binding of all type-specific antibodies was significantly lower with >2 M GuHCl. Concentrations of 1 M GuHCl resulted in <20% change in mAb binding compared to untreated VLPs for all types. Thus, 1 M GuHCl at 37 °C for 15 min were selected as the dissociating conditions for the avidity assay. These same conditions also resulted in <10% change in mAb binding between treated versus untreated wells on the 5 additional VLP types, (HPV 31, 33, 45, 52, and 58) on M9ELISA plates (Fig. 3).

3.2. Determination of avidity indices across multiple dilutions within the detectable range of the assay

The AIs for dilutions of six samples for each HPV type are shown in Table 1. The samples showed a wide range of AI (0.2–0.8), however, the AIs were similar across dilutions for any

given HPV VLP type (Table 1). Median %CV for HPV 6 was 7% (range 2–23%), HPV 11 was 4% (2–21%), HPV 16 was 5% (1–7%), and HPV 18 was 8% (1–24%).

3.3. Evaluation of assay variation using serum with a wide range of anti-body titers

Twenty four de-identified residual serum samples with a wide range of Ab titers were tested to evaluate assay precision in calculating AIs over multiple runs. Overall, the median inter-assay variation across the three independent experiments were <10% for all types, with a median of 5% for HPV 6 (range 1–23%), 6% for HPV 11 (1–17%), 7% for HPV 16 (4–30%), and 5% for HPV 18 (2–21%).

Fig. 4 shows the distribution of the AIs for HPV 6, 11, 16, and 18. The average AIs ranged from 0.07 to 0.80 for HPV 6, 0.15 to 0.84 for HPV 11, 0.29 to 0.85 for HPV 16, and 0.16 to 0.82 for HPV 18 for this set of samples. The antibody titers of these samples had been previously determined by M4ELISA. The median titer for HPV 6 was 18.9 AU/mL (range of 3.6–1134.1 AU/mL), HPV 11 was 44.2 AU/mL (range of 0.7–1587.8 AU/mL), HPV 16 was 89.0 IU/mL (range of 1.6–5665.4 IU/mL), and HPV 18 was 33.0 IU/mL (range of 0.9–3411.8 IU/mL). There was moderate correlation between antibody titers and AI for HPV 11, 16, and 18 (p-value = 0.0160, 0.0004, and 0.0002, respectively, Fig. 5B–D); however, HPV 6 showed no correlation (p-value = 0.1202, Fig. 5A).

4. Discussion

In this report we describe the development and validation of a high throughput multiplex electrochemiluminescent avidity ELISA for studying IgG antibody response to HPV types 6, 11, 16, and 18. We established a concentration of GuHCl and assay conditions that minimally impacts HPV VLP conformation for multiple types, while allowing disruption of low avidity antibodies as shown by the wide range of AIs for each type. Unlike Dauner et al., where concentrations of up to 3 M GuHCl were used, in our study VLP conformation was adversely affected at concentrations higher than 2 M (Dauner et al., 2012). The reason for this difference is unclear. It could be due to differences in VLP production methods or the higher amounts of VLP used per well by Dauner et al. (2012) which may have allowed for use of higher concentrations of chaotropic agent before a conformational change was observed. Two bead-based multiplex avidity assays have also been published (Scherpenisse et al., 2013; Sankaranarayanan et al., 2016); however, the effect of the chaotropic agent on the VLP or protein structure (GST-tagged partial L1) was not discussed.

We calculated AI based on a standard curve, which is a method commonly used in other published avidity ELISA assays (Anttila et al., 1998; Licciardi et al., 2012; Almanzar et al., 2013; Scherpenisse et al., 2013; Boxus et al., 2014). As long as results fell within the limit of detection the AI for individual samples was independent of dilution (Table 1), permitting use of a single serum dilution for AI determination. Overall, assay variation was low. The M4-AvELISA shares the advantages of the M4ELISA including having a large dynamic range, low background, and plate stability up to 12 months (Panicker etal., 2015). Moreover, because of the inherent wide dynamic range of our assay, it would be possible to look at avidity of cross reactive antibodies, something which other studies have cited. One inherent limitation of all avidity assays, regardless of the platform used, is the assumption that

antibody binding does not locally alter the structure of the protein at or near the binding site. However, for an overall analysis of avidity, the net effect of the antibodies binding to VLPs is sufficient for comparison of trends with time, correlations with titers, and changes due to vaccine dose(s) and intervals.

Studies of the antibody response to the 9-valent HPV vaccine will require methods to evaluate the avidity for 5 additional types. While we did not validate our assay for the 5 additional HPV types using serum, we have shown that assay conditions selected here can be used to simultaneously determine the avidity for all types included in the 9-valent vaccine. We did note some variation in conformational monoclonal antibody binding following treatment with GuHCl, especially at concentrations higher than 2 M. However, there was minimal effect on confirmation of types 31, 33, 45, 52 and 58 at 1 M conditions.

Understanding antibody avidity provides insight into the quality of antibodies produced in response to vaccination or natural infection. Though avidity is correlated with neutralization of pathogens, the importance of antibody avidity for protection against HPV remains to be determined. A few recent studies have shown that antibody avidity is higher in vaccinated versus naturally infected sera (Scherpenisse et al., 2013), similar avidity indices are maintained following either 2 doses versus 3 doses of the bivalent HPV vaccine (Boxus et al., 2014), and antibody avidity to HPV 16 increased over time up to 12 months following vaccination with either the bivalent (Kemp et al., 2012) or quadrivalent HPV vaccine (Herrin et al., 2014). There is limited evidence comparing HPV antibody avidity with neutralizing antibody titer. In this report, we found a moderate correlation between AI and antibody titer, however, this was a limited sample set and vaccination status was unknown. No neutralization assays were performed to determine a correlation of neutralizing titer with AI. Further study is needed to determine the relationship between HPV antibody avidity, antibody titer, and neutralization. The M4-AvELISA (and planned M9-AvELISA) will allow high-throughput multi-valent testing of large sample sets.

Acknowledgments

Funding

Source of research support is provided by the Centers for Disease Control and Prevention through the Prevention and Public Health Fund.

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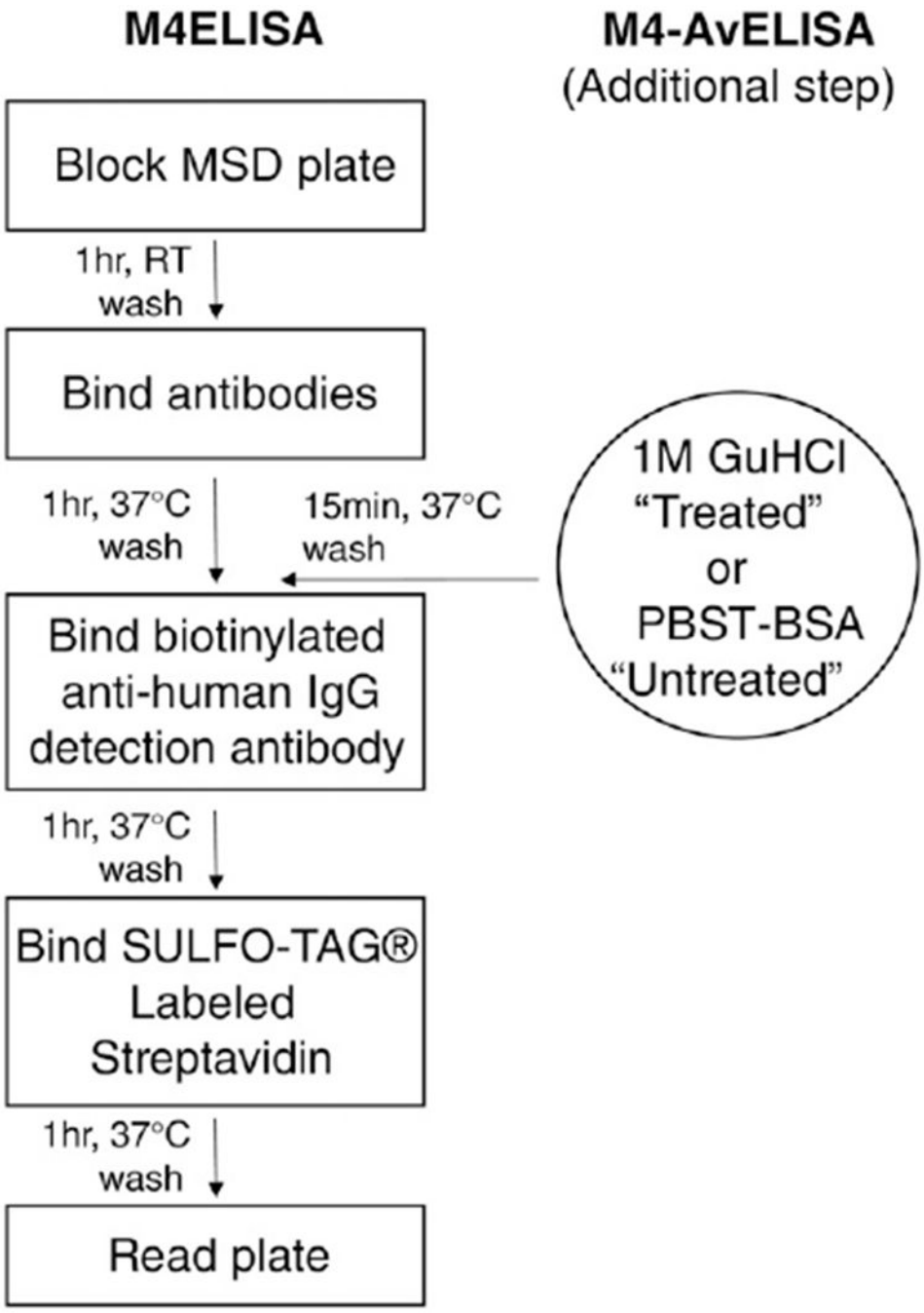

Fig. 1.
Schema of M4-AvELISA.

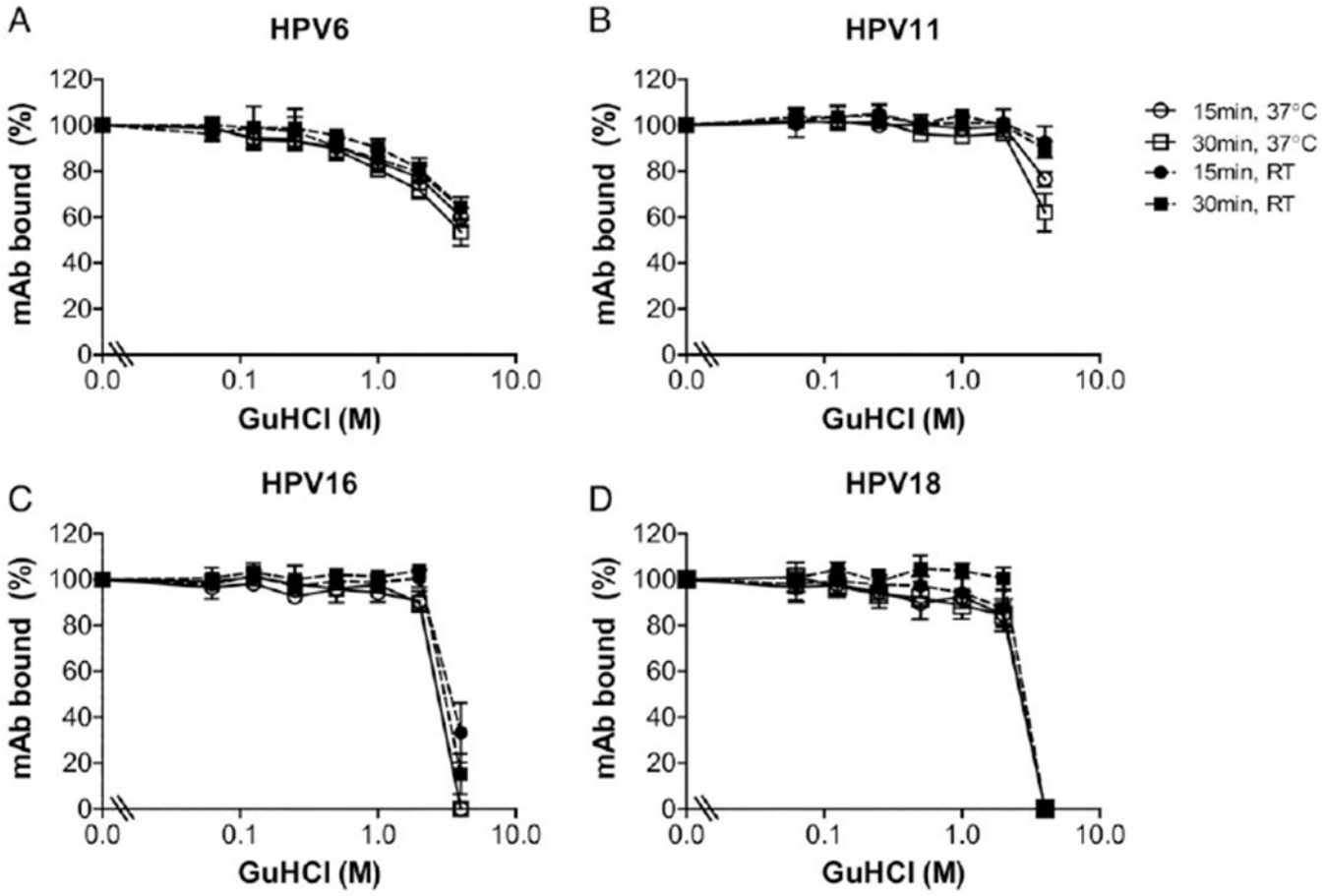

Fig. 2.

Monoclonal Ab binding to HPV 6 (A), HPV 11 (B), HPV 16 (C), and HPV 18 (D) following treatment with various concentrations of guanidine hydrochloride treatment (GuHCl) at either 37 °C (solid lines) or room temperature (RT, dashed lines) for either 15 min (open circles) or 30 min (open squares). Data shown is a representative experiment.

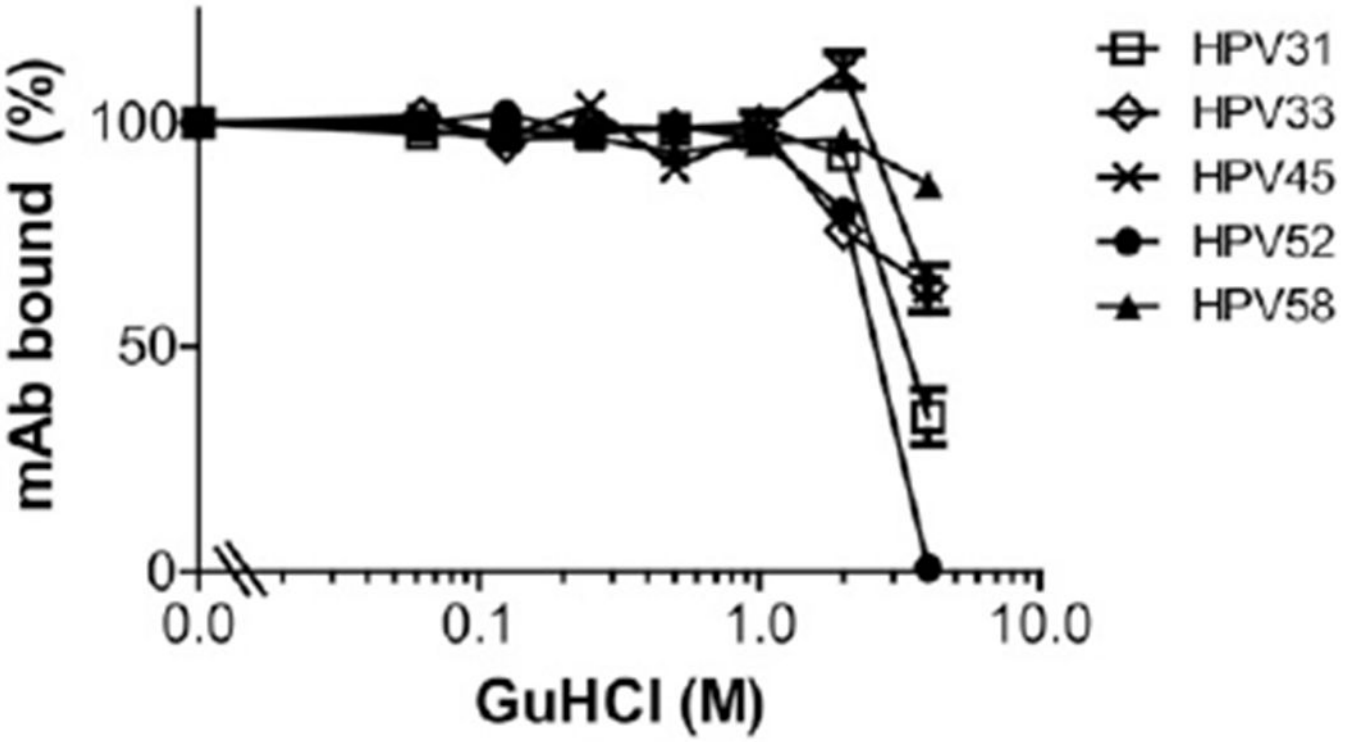

Fig. 3.

Effect of guanidine hydrochloride at various concentrations for 15 min at 37 °C on 5 additional HPV types (HPV 31, 33, 45, 52, and 58). Data shown is a representative experiment.

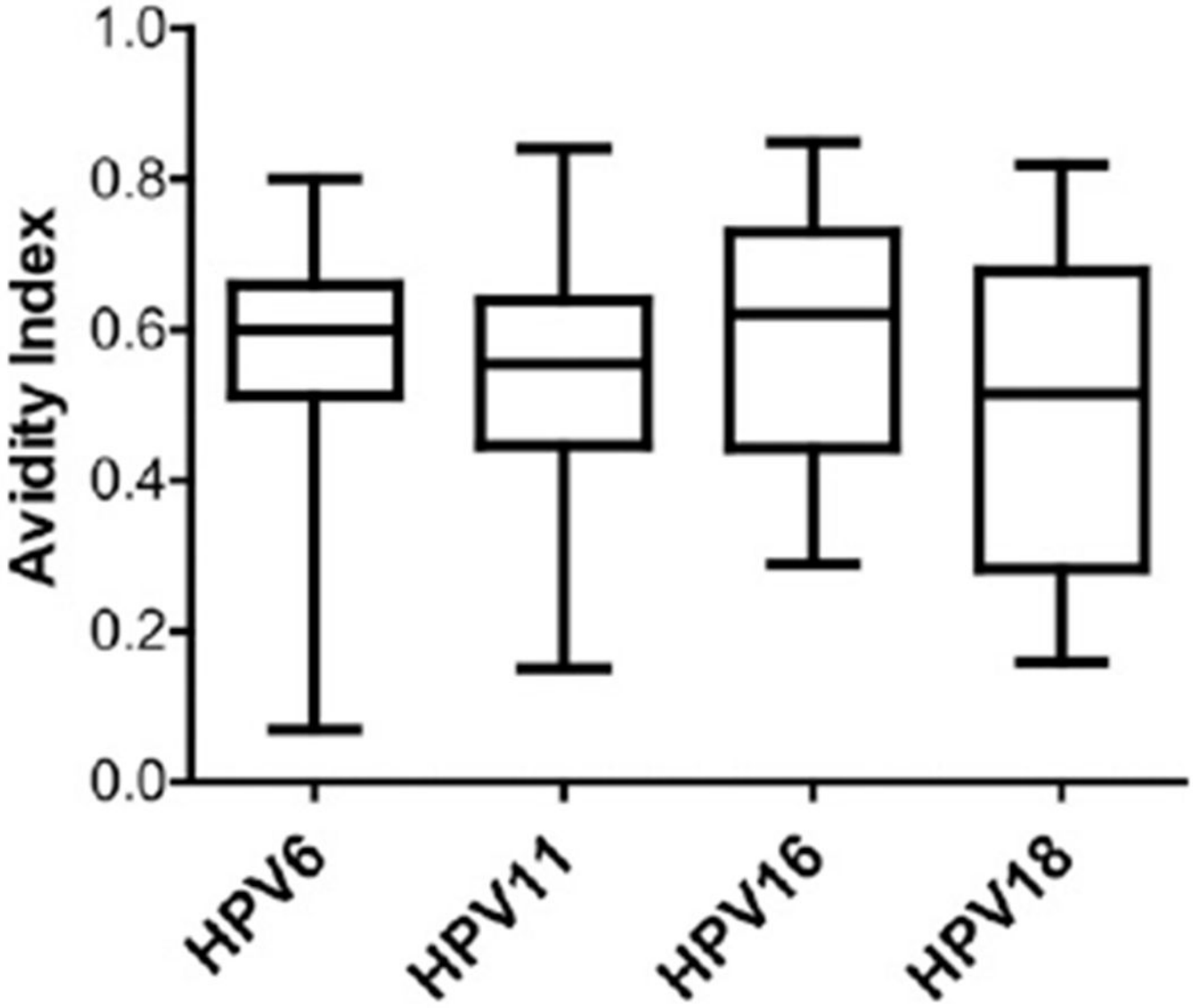

Fig. 4.
Avidity indices for 24 samples for HPV 6, 11, 16, and 18.

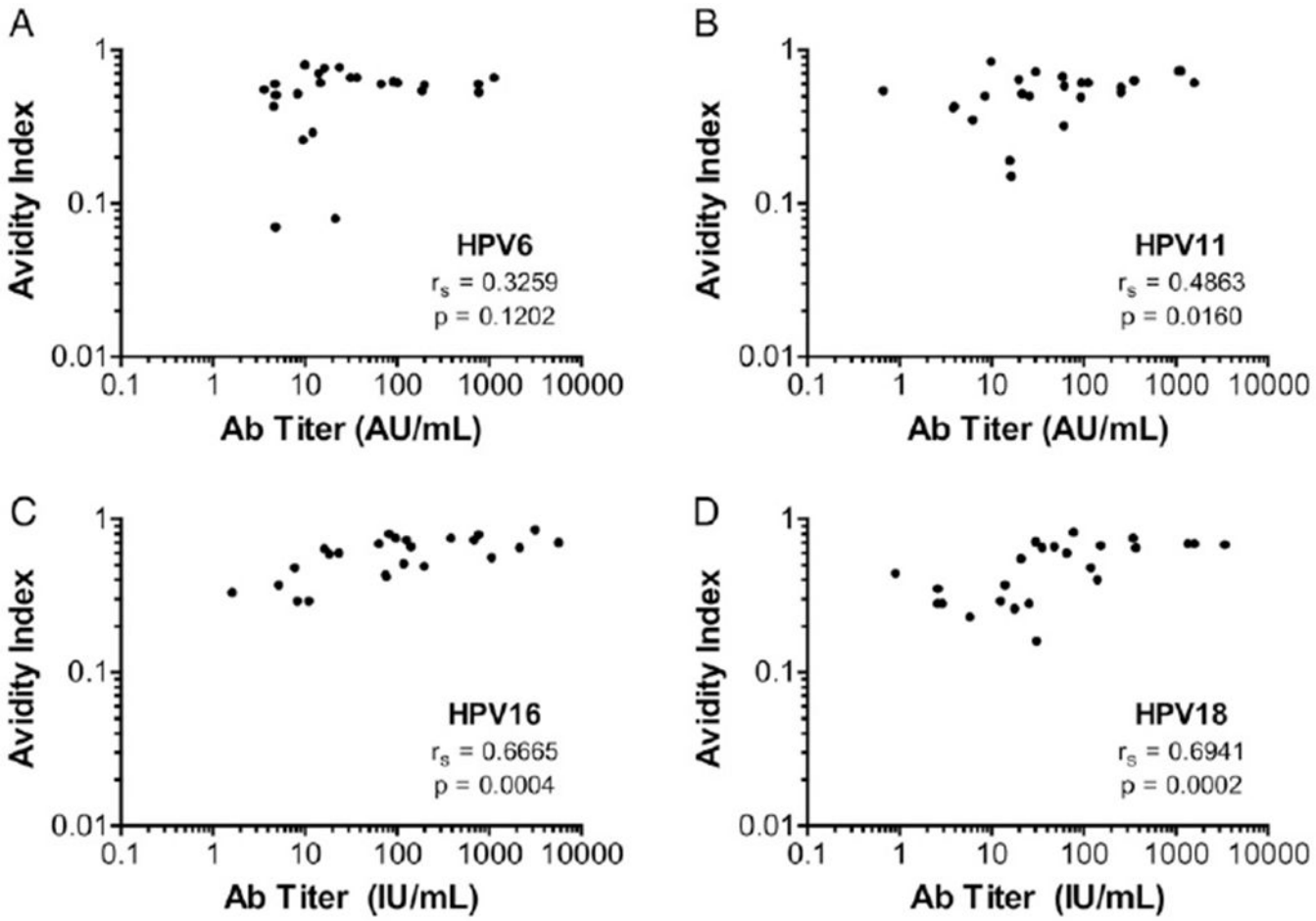

Fig. 5.

Correlation between avidity index and antibody titer for HPV 6 (A), HPV 11 (B), HPV 16 (C), and HPV 18 (D).

Table 1

Avidity indices for HPV 6, 11, 16 and 18 for 6 samples across multiple dilutions.

Sample #	Dilution (1:)	HPV6	HP11	HPV16	HPV18
1	**31.6**	0.6	0.2	0.8	0.4
	100	0.6	0.3	0.8	0.5
	316	0.6	0.3	0.9	0.5
	1000	0.6	0.4	0.8	0.5
2	**100**	0.7	0.7		0.7
	316	0.6	0.7	0.8	0.7
	1000	0.6	0.7	0.8	0.7
	3160	0.6	0.7	0.8	
3	**31.6**	0.1	0.2	0.4	0.5
	100	0.1		0.4	0.4
	316			0.4	0.5
	1000			0.4	
4	**1000**		0.7		
	3160	0.7	0.7	0.8	0.7
	10,000	0.8	0.7	0.8	0.7
	31,600	0.7	0.7	0.8	0.7
5	**1000**	0.8	0.9		0.8
	3160	0.7	0.8	0.9	0.8
	10,000	0.6	0.7	0.8	0.7
	31,600	0.6	0.8	0.8	0.8
6	**31.6**	0.6	0.8	0.4	0.5
	100	0.6	0.9	0.4	0.6
	316	0.7	0.8		0.8
	1000	0.6			

Note: Black boxes indicate where the RLUs for that particular dilution were outside of the limit of detection and were therefore not used for calculating the avidity index.