

Quantifying Specific Antibody Concentrations by Enzyme-Linked Immunosorbent Assay Using Slope Correction

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Received 23 December 2005/Returned for modification 27 February 2006/Accepted 8 May 2006

Assessing the magnitude of an antibody response is important to many research and clinical endeavors; however, there are considerable differences in the experimental approaches used to achieve this end. Although the time-honored approach of end point titration has merit, the titer can often be misleading due to differences in how it is calculated or when samples contain high concentrations of low-avidity antibodies. One frequently employed alternative is to adapt commercially available enzyme-linked immunosorbent assay kits, designed to measure total antibody concentrations, to estimate antigen-specific antibody concentrations. This is accomplished by coating the specific antigen of interest in place of the capture antibody provided with the kit and then using the kit's standard curve to quantify the specific antibody concentration. This approach introduces considerable imprecision, due primarily to its reliance on a single sample dilution. This "single-point" approach fails to address differences in the slope of the sample titration curve compared to that of the standard curve. Here, we describe a general approach for estimating the effective concentration of specific antibodies, using antisera against foot-and-mouth disease virus VP1 peptide. This was accomplished by initially calculating the slope of the sample titration curve and then mathematically correcting the slope to that of a corresponding standard curve. A significantly higher degree of precision was attained using this approach rather than the single-point method.

Enzyme-linked immunosorbent assays (ELISAs) are among the most commonly employed laboratory techniques due to their flexibility, sensitivity, low cost, and ease of automation. ELISAs are used clinically to assess the magnitude of an immune response for a variety of purposes, including diagnosing disease based upon seroconversion to an infectious agent, assessing the course/status of an ongoing clinical disease, and predicting protective immunity against infectious diseases. In vaccine research, ELISA titers are often used to identify and/or map neutralizing epitopes and to establish correlates of immune protection. In many instances, quantifying the antigen-specific antibody concentration is desirable.

Customarily, end point titration has been used to quantify the magnitude of an antibody response, resulting in an assessment of "titer." This simple technique suffers from two significant shortcomings. First, there is no universally accepted method for assigning titer, resulting in imprecision and ambiguity when results obtained by different laboratories are compared. Second, samples containing high concentrations of low-avidity antibodies are often assigned artificially high titers (3, 7). The resulting titers may be misleading, due to the minimum avidity requirements needed for protection (1). Thus, titers can be unreliable in predicting protection against an infectious disease as well as in assessing the magnitude of immune responses.

An alternative to end point titration has emerged over the past decade upon commercialization of capture ELISA kits. These kits use a sandwich ELISA format to quantify the concentration of a

variety of soluble proteins, including cytokines, hormones, growth factors, and antibodies. Generally, these kits are accurate and precise in that both the solid-phase capture and detection antibodies are well characterized and bind to their target antigens in a predictable and reproducible manner. In many research publications, substitution of the solid-phase capture antibody with a coated antigen has been used to determine antigen-specific antibody concentrations. In many instances, this approach relies on a single sample dilution's optical density (OD) falling within the bounds of the standard curve. This "single-point" interpolation is fundamentally flawed (8) in that it does not take into account differences in the slope of the sample titration curve compared to that of the standard curve.

It is generally accepted that the slope of an antibody titration curve is proportional to the average antibody avidity (5, 9). To incorporate slope correction into the indirect ELISA system, a mathematical model of avidity differences between samples was developed based on the law of mass action (2, 4). This approach yielded a considerably more precise determination of specific antibody concentration.

MATERIALS AND METHODS

Animals and immunizations. Five-week-old Yorkshire pigs of each gender were obtained from Tufts University School of Veterinary Medicine (North Grafton, MA). After a 1-week acclimatization period, each pig was anesthetized (4.4 mg telazol/kg of body weight, 2.2 mg/kg ketamine, and 2.2 mg/kg xylazine) and then vaccinated intramuscularly with 100 μ g of a foot-and-mouth disease virus (FMDV) peptide vaccine (UBITh [10]). Whole blood was collected on a weekly basis and centrifuged at 1,000 \times g, and sera were stored at -20°C until assayed. An identical booster immunization was given 6 weeks later, and the animals were sacrificed at week 9 by administration of Euthasol (Delmarva Laboratories Inc., Midlothian, VA) at 0.22 ml/kg (85.8 mg/kg sodium pentobarbital and 11 mg/kg sodium phenytoin). All animal protocols were approved by the University of Connecticut's Institutional Animal Care and Use Committee prior to the initiation of procedures.

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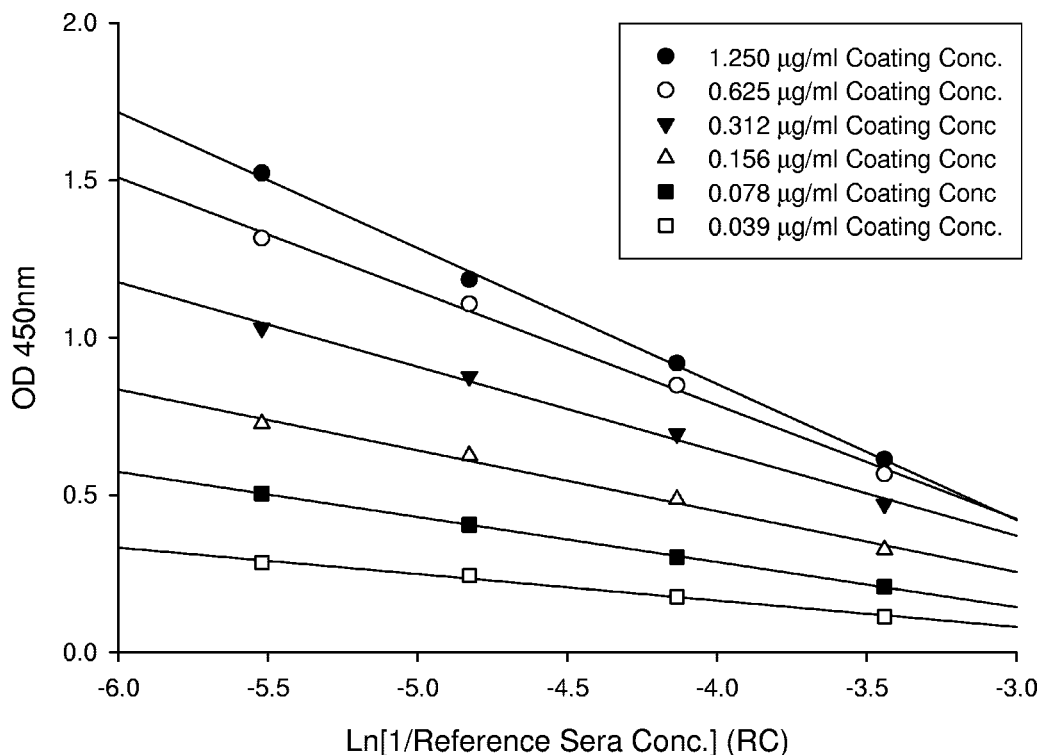


FIG. 1. The relationship between the optical density and dilution of a series of standard curves at different coating concentrations.

Immunoassays. Plates were coated in one of two ways. On plates receiving the standard curves, affinity-purified, anti-swine immunoglobulin G (IgG) capture antibodies (Bethyl Laboratories, Montgomery, TX) were diluted in coating buffer (0.05 M sodium carbonate, pH 9.6) and 50 µl was applied to flat-bottomed 96-well Immulon IV HBX microtiter plates (Thermo Labsystems, Franklin, MA) at concentrations from 1.25 µg/ml to 0.039 µg/ml representing the variable CC. On plates receiving samples, the capture antibody was substituted with 50 µl of the antigen of interest (FMDV peptide vaccine [UBIth] or bovine serum albumin [BSA] control) at 10 µg/ml in coating buffer. Both sets of plates were tightly sealed with Parafilm and incubated overnight at room temperature (RT).

Standard curves were produced from pig reference serum provided with the kit and serially (log₂) diluted from 250 ng/ml to 31.25 ng/ml. The diluted standards were applied to plates previously coated with affinity-purified anti-swine IgG coated at 50 µl/well.

Sample curves were serially (log₂) diluted from pig sera at 1:2,000 to 1:8,000 and added to antigen-coated plates. From this point forward, both types of plates were treated identically.

Plates were washed with wash buffer (50 mM Tris, 0.14 M NaCl, 0.05% Tween) three times using 125 µl of buffer, with shaking, using a Bio-Tek EL-403 plate washer (Bio-Tek Instruments, Inc., Winooski, VT) between each step. Plates were blocked with 100 µl/well of a Tris-buffered saline solution containing 0.1% BSA for 30 min at RT. Samples and standards were serially diluted (log₂) in Tris-buffered saline (50 mM Tris, 0.14 M NaCl, 1% BSA, 0.05% Tween, pH 8.0) and incubated at RT for 1 h.

After the plates were washed, a horseradish peroxidase-conjugated goat anti-pig IgG (Bethyl) was applied at a concentration of 1:20,000 (50 µl/well) and incubated for 1 h at RT. Plates were washed, and 50 µl/well of 3,3',5,5'-tetramethylbenzidine (Kirkegaard-Perry, Gaithersburg, MD) microwell peroxidase substrate was added, resulting in the development of a colored product. Plates were allowed to develop at RT for 10 min, at which time the reaction was stopped upon addition of 50 µl/well of 2 N H₂SO₄. The OD of each well was determined by measuring the absorbance at a λ value of 450 nm using a Bio-Tek EL-311 microplate autoreader (Bio-Tek Instruments, Inc., Winooski, VT).

To determine the slope of each sample's titration curve, a first-order quadratic formula was fitted to characterize the relationship between OD and log-transformed dilution. Only curves with r² values of ≥0.99 were used in subsequent analyses. Standard curves were generated using pig reference serum (Bethyl) containing a known concentration of antibodies. The reference serum was di-

luted from a 1-µg/ml stock solution at concentrations of 250 ng/ml, 125 ng/ml, 62.5 ng/ml, and 31.25 ng/ml, defining the variable RC. The coating concentration was optimized in order to obtain a linear slope regression.

Data analysis. (i) Single-point analysis. Specific antibody concentrations were determined by selecting sample dilutions that fell within the upper and lower bounds of the standard curve (generated using the capture ELISA system). The concentrations were calculated using a single-point interpolation without slope correction. The resulting value was multiplied by the dilution factor of the sample to correct for the final concentration.

(ii) Slope-corrected analysis. In order to determine the slope [m_(CC)] and intercept [b_(CC)] of each standard curve, the RC and CC values were substituted into equation 1. This relationship is illustrated by the four curves shown in Fig. 1.

$$m_{(CC)} = \frac{\sum \{[\ln(RC) - \overline{\ln(RC)}][OD_{(CC)} - \overline{OD_{(CC)}}]\}}{\sum \{[\ln(RC) - \overline{\ln(RC)}]^2\}}$$

$$b_{(CC)} = \overline{OD_{(CC)}} - m_{(CC)} \times \overline{\ln(RC)} \tag{1}$$

The slopes and intercepts determined from equation 1 were then substituted into a least-squares formula and plotted against the coated antigen concentrations (CC), yielding the slope and intercept matrix:

$$m_{\text{SlopeMatrix}} = \frac{\sum \{[\ln(CC) - \overline{\ln(CC)}][m_{(CC)} - \overline{m_{(CC)}}]\}}{\sum \{[\ln(CC) - \overline{\ln(CC)}]^2\}}$$

$$b_{\text{SlopeMatrix}} = \overline{m_{(CC)}} - m_{\text{SlopeMatrix}} \times \overline{\ln(CC)} \tag{2}$$

$$m_{\text{InterceptMatrix}} = \frac{\sum \{[\ln(CC) - \overline{\ln(CC)}][b_{(CC)} - \overline{b_{(CC)}}]\}}{\sum \{[\ln(CC) - \overline{\ln(CC)}]^2\}}$$

$$b_{\text{InterceptMatrix}} = \overline{b_{(CC)}} - m_{\text{InterceptMatrix}} \times \overline{\ln(CC)}$$

This resulted in a log-linear relationship between the coating concentration and the slope/intercept (Fig. 2). The relative coating concentration of the predicted, slope-matched standard curve (CC_{STDCorr}) was then determined by substituting the slope of the sample titration curve into equation 3.

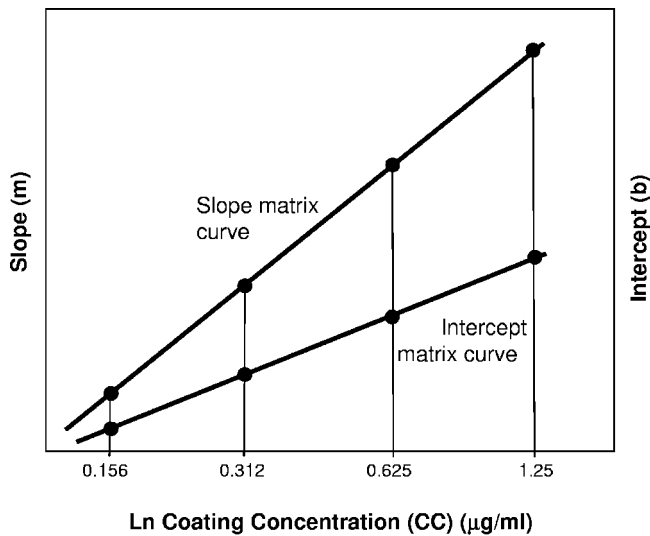


FIG. 2. Regression of the slopes and intercepts (step 1) versus the coating concentrations (step 2).

$$CC_{STDcorr} = \frac{(m_{samp} - b_{SlopeMatrix})}{m_{SlopeMatrix}} \tag{3}$$

This term was used to determine the intercept ($b_{STDcorr}$) as follows:

$$b_{STDcorr} = (m_{InterceptMatrix} \times CC_{STDcorr}) + b_{InterceptMatrix} \tag{4}$$

Each sample's effective specific antibody concentration was then calculated, using any OD reading from the sample dilution curve, as follows:

$$Conc_{STD} = e^{\left(\frac{OD_{Samp} - b_{STDcorr}}{m_{Samp}}\right)} \tag{5}$$

The dilution factor corresponding to the OD used in equation 5 was then substituted into equation 6 to correct for each sample's dilution:

$$Conc_{Samp} = \left(\frac{Conc_{STD}}{Dil_{Samp}}\right) \tag{6}$$

RESULTS

Significant heterogeneity was observed in titration curve slopes for antisera collected from FMDV peptide-immunized pigs (Fig. 3). By applying the formulae in equations 1 to 5, these differences in slope were corrected, yielding "effective antibody concentrations." The results obtained using this method were then compared to those obtained using the single-point method. Significantly lower coefficients of variation (CVs) were found using the slope correction approach rather than the single-point method. ($P < 0.01$) (Fig. 4).

DISCUSSION

End point titration estimates of titer frequently lead to ambiguous and imprecise results. Thus, many investigators have elected to express the magnitude of humoral immune responses in terms of specific antibody concentration. This is accomplished by substituting the coated capture antibody, provided in commercially available ELISA kits, with the antigen of interest. This approach generally results in the OD corresponding to a particular sample's dilution being interpolated on a standard curve generated using the capture antibody. Here, we show that this approach leads to considerable imprecision, as it does not address differences in slope that inevitably occur between a given sample's dilution curve and the standard

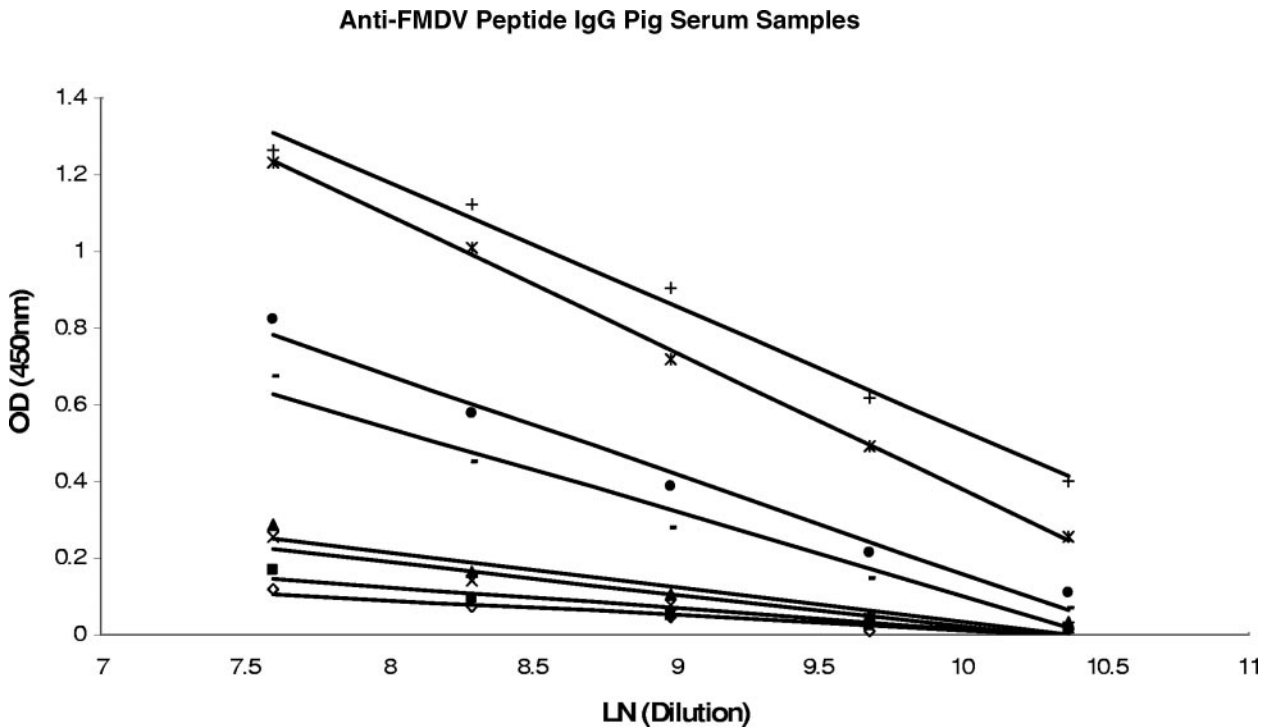


FIG. 3. Serum IgG responses against an FMDV peptide vaccine (UBITH) showing heterogeneity in slopes. Each symbol represents an individual animal.

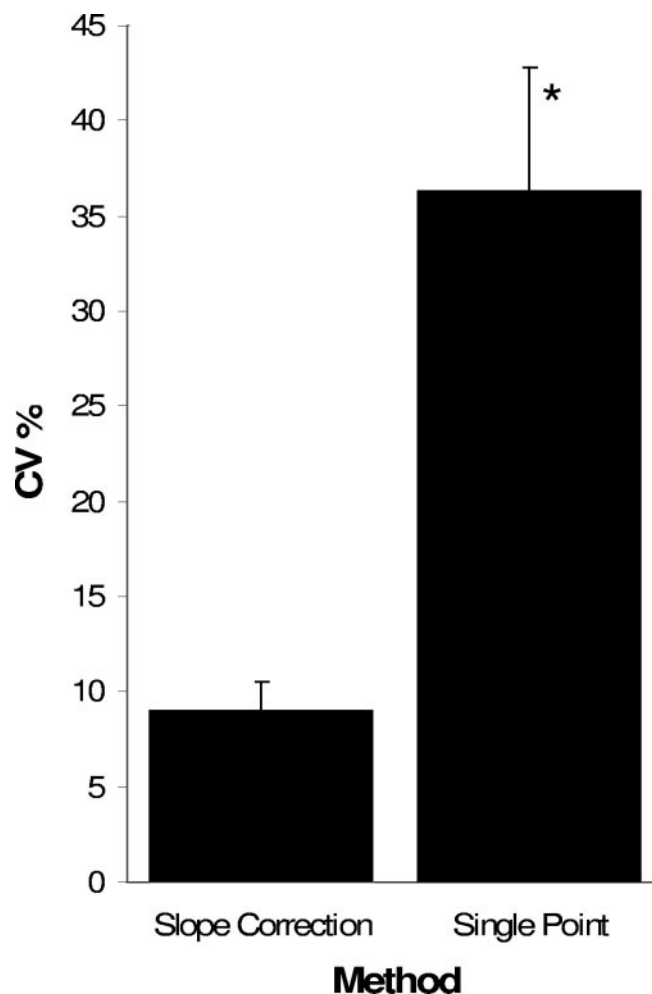


FIG. 4. Comparison of CVs using the single-point versus the slope correction method. The single-point method has a significantly higher CV (*, $P < 0.01$) than the slope correction method.

curve. To correct for this deficiency, the slopes of both curves were mathematically modeled and the standard curve was slope matched to the sample curve, thereby providing an estimate of the sample's concentration.

Lemke et al. recently described an alternative approach for estimating specific antibody concentrations by using an antibody depletion technique (6). Specific antibodies were depleted in a stepwise fashion by transferring the unbound antibody population from one antigen-coated well to the next. The specific antibody concentration was then determined by calculating the difference between antibody concentration before

and after depletion. Although this method is conceptually sound, it was unreliable in our hands due to unavoidable loss of sample at each transfer step. In addition, this approach may overestimate the specific antibody concentration due to the nonspecific binding that occurs at each step (data not shown). Further refinement of this approach may be useful in defining an "absolute" specific antibody concentration for a single sample that could then be used as a reference standard. Pairing this method with the slope correction approach described here could ultimately improve both the accuracy and precision of ELISAs.

Although the slope-matching technique described herein is slightly more cumbersome than either end point titration or the single-point interpolation approach using commercial capture ELISA kits, it is considerably more precise. Adopting this approach will lead to better agreement between research groups in assessing the magnitude of antigen-specific antibody responses.

ACKNOWLEDGMENTS

We thank Debra Rood for critical review of the manuscript, Alan Walfield, United Biomedical Inc., for providing the FMDV peptide, and Roger Zemek for assistance with animal care and handling.

This work was supported in part by USDA grants 58-1940-0-007 and NRICGP 2002-02833.

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