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Short Communication

Estimating Duration of Infection with Avidity Assays: Potential Limitations and Recommendations for Improvement

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Abstract: Recent infections often have higher pathogen loads. The number of recent infections can therefore be used to estimate transmission rates in a host population. Antibody avidity assays are an emerging technique to infer infection age in both domestic and wild animals. These assays have the potential to supplant intensive mark-recapture efforts for identification of recent infections, but their results may be confounded by antibody titer. We examined the effectiveness of an avidity assay for identifying recent infections of Sin Nombre virus, a hantavirus in North America that establishes a chronic infection in deer mice (*Peromyscus maniculatus*). We found that assay performance statistics such as sensitivity, specificity, and positive predictive value for low avidity scores were significantly improved when we accounted for antibody titer in the analyses. Without accounting for titer, avidity assays may classify samples with low titers as recent infections regardless of actual infection history, thereby overestimating the number of recent infections in a population and inflating estimates of transmission rates and/or human exposure risk. We recommend that antibody titers meet a minimum threshold for use in avidity assays, and we emphasize the importance of considering titer and dilution in the validation of newly developed avidity assays.

Keywords: wildlife disease, avidity assays, infection history, Sin Nombre virus

In many host–pathogen systems, animals with recent infections have higher pathogen loads. This pattern is observed for a wide variety of vertebrate hosts (e.g., Wiger 1977; Hutchinson et al. 1998; Atkinson et al. 2001; Hardestam et al. 2008). Thus, the proportion of infections that are recent can be used to infer transmission rates within a host population. For zoonotic diseases, high pathogen loads associated with recent infections in wildlife may also elevate the exposure risk for humans living in close proximity to infected animals (McCormick and Fisher-Hoch 2002). The rapid identification of host populations with high proportions of recent infections is therefore of great interest, as such populations may be associated with future human disease outbreaks. Estimating the duration of infection in wildlife populations has traditionally been accomplished by monitoring an individual's antibody status during extensive mark-recapture studies; however, these studies are often laborious and expensive. In addition, many hosts of zoonotic pathogens such as bats and rodents have short life spans and low recapture rates; these conditions make it difficult to detect an infection at sufficient temporal resolution to infer when it was acquired (e.g., Douglass et al. 2007; Previtali et al. 2010).

To surmount the difficulties associated with mark-recapture studies, assays to measure antibody binding

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strength have been developed to estimate duration of infection for several zoonotic pathogens in both domestic and wild vertebrate hosts (e.g., Gavrilovskaya et al. 1993; Sager et al. 2003; Safronetz et al. 2006; Kinnunen et al. 2007; Kinnunen et al. 2011). These assays have been a common medical detection tool for over 20 years (e.g., Hedman and Seppälä 1987; Guy et al. 2009), but they have only recently been applied in ecological contexts. The assays are based on the principle that antibody avidity (defined as the overall binding strength of an antibody to a specific antigen) increases as the immune response progresses through time (Janeway et al. 2005). Antibody avidity assays are an appealing tool for surveying short-term infection history in animals because, once validated, they do not require serial samples from the same individual. In addition, the assays require a limited amount of serum that can be collected nondestructively (e.g., from protected species). Avidity assays therefore hold great promise for monitoring the prevalence of recently infected hosts. Applications for avidity results include predicting outbreaks of disease within a protected species or transmission events associated with wild animals living in close proximity to humans or livestock. The assays are also an easy extension of many ongoing assessments of human exposure risk (Lemon et al. 2008; Carver et al. 2010).

An implicit assumption of these assays is that high avidity antibodies will be maintained in hosts with older infections. However, some studies have suggested that avidity assays may also be influenced by the amount of antibody in the sample, especially when samples are only tested at a single dilution (Hedman and Seppälä 1987; Kneitz et al. 2004). In this study, we evaluate the accuracy of an avidity assay for determining the age of Sin Nombre virus (SNV) infection in deer mice (*Peromyscus maniculatus*). We also provide recommendations for improving the accuracy of this assay and offer advice for the development of avidity assays in other host–pathogen systems.

SNV is a hantavirus of significant public health concern in North America (Young et al. 2000). It is primarily hosted by the ubiquitous deer mouse (Childs et al. 1994), where it establishes a chronic infection with no obvious pathologic symptoms (Botten et al. 2003). Infected animals will thus produce antibodies for life, and presence of antibody is commonly used as a marker of current infection in individuals (Botten et al. 2003). We analyzed a total of 71 serum samples from 56 adult deer mice for this study. These samples came from our research group's long-term mark-recapture effort in central Utah from 2005 to 2009. Deer mice were live-trapped as previously described (Lehmer et al. 2008; Previtali et al. 2010) in May and September at nine sites. In addition, two sites were sampled at more frequent intervals (every 35 days). During each sampling period, we marked deer mice with unique ear tags, collected 200 μl of blood, and released animals at the point of capture. Sera were then screened for SNV-specific Immunoglobulin G (IgG) antibodies with an enzyme-linked immunosorbent assay (ELISA) using a recombinant, affinity-purified SNV-nucleocapsid antigen (Feldmann et al. 1993; Otteson et al. 1996). To determine endpoint antibody titers, seropositive samples were tested at increasing dilutions from 1:100 to 1:51200. Titer was defined as the reciprocal of the last dilution with a positive ELISA result; thus, samples with higher titers contain higher SNV-specific antibody levels.

Avidity assays were also performed on IgG antibody-positive samples at a single dilution (1:100) as previously described (Safronetz et al. 2006). Like most avidity assays, this assay relies on a weak protein denaturant (in this case, 35 mM diethylamine), which disrupts binding of low avidity antibodies, but should not disrupt high avidity antibodies. Avidity scores were calculated by dividing the optical density of a sample treated with denaturant by its optical density without the denaturant (Hedman and Seppälä 1987; Safronetz et al. 2006). These scores range from zero to one. For SNV, low avidity scores (<0.4) indicate recent infection (35 days or less), and high avidity scores (>0.6) indicate older infections. Samples with scores between 0.4 and 0.6 are considered ''intermediate'' and cannot be classified as either recent or older (Safronetz et al. 2006).

We also determined duration of infection using traditional mark-recapture methods. Deer mice were considered to have older infections (>35 days) after antibodies to SNV were detected in two sampling periods spaced at least 35 days apart. For older infections, only the second sample was analyzed for titer and avidity assays ($N = 60$ samples from 46 individuals) because these infections were definitely at least 35 days old at the time of the second sample. Detection of recent infections was less definitive because of our sampling intervals and the animal's immune response to the infection. Deer mice that seroconverted (negative on first sample; positive on second) within a 35-day sampling interval may have had recent infections (<35 days, $N = 11$). Alternatively, these animals could have been infected upon their initial capture but did not have a detectable antibody response, which may not appear until

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14–21 days post infection (Botten et al. 2000). Thus, these infections may have been up to 56 days old. We recognize that our classification of recent infections is less certain and therefore draw conclusions concerning these animals cautiously. For these animals, the first antibody-positive samples were analyzed for titer and avidity.

A Bayesian analysis was performed in SISA (Uitenbroek 1997) to estimate base rates and predictive values. These measures are sensitive to the "prevalence" of recent infections (i.e., the percent of infected animals that seroconverted within a 35-day interval), which varied from 0 to 23.0% over five trapping periods. The average value for the percentage of recent infections (11.6%) was used for this analysis. Wilcoxon–Mann–Whitney tests were used to compare titers of animals with "recent" versus "older" infections or "high" versus "low" avidity scores. Spearman's rank order correlations were used to determine the relationship between avidity scores and titer and also the relationship between avidity scores and sample dilution. These analyses were performed with R 2.13.1 (© 2011 The R Project for Statistical Computing, http://www.r-project.org/).

The avidity assay correctly classified 72% of infected animals in this study when compared to data from recapture histories (51 of 71; Table 1). Eight of 11 samples from recent infections had low avidity scores, thus the assay sensitivity (the probability that a recent infection has a low avidity score) was 72.6%. Forty-three of 60 samples from older infections had high avidity scores, thus, the specificity (the probability that an older infection has a high avidity score) was 71.7%. A Bayesian analysis was conducted using

Table 1. Correspondence between avidity scores and capture history for 71 samples from 56 adult deer mice

Avidity score	Infection history (estimated by capture history)	
	Older	Recent
High (>0.6)	N = 43 Titer = 12355[a]	**N = 2** **Titer = 27713[a]**
Intermediate (0.4–0.6)	N = 6 Titer = 5702[a]	N = 1 Titer = 25600
Low (<0.4)	**N = 11** **Titer = 662[a]**	N = 8 Titer = 2263[a]

Values in bold indicates a discrepancy between avidity score and capture history.

N number of samples tested.

[a]Titer reported is a geometric mean.

the assay's sensitivity and specificity and the overall prevalence of recent infections to predict the false positive rate of 24.8% (the rate at which older infections are classified as recent infections) and the false negative rate of 3.1% (the rate at which recent infections are classified as older infections; Table 2). The Bayesian analysis also generated measures of predictive value (i.e., the probability that a low avidity score indicates a recent infection and vice versa). The negative predictive value was 95.4%, indicating that samples with high avidity scores are extremely likely to have come from older infections. In contrast, the positive predictive value was far lower, at 25.3%, indicating that samples with low avidity scores have a 1 in 4 chance of indicating a recent infection (Table 2). Indeed, over half of the low avidity (<0.4) samples in our study (11 of 19) came from animals with definitively older infections. This result is in stark contrast to previous studies in which animals with infections older than 35 days never had low avidity scores (Safronetz et al. 2006).

To help explain the low positive predictive value for avidity scores and develop potential methods to improve the assay's performance, we investigated the relationship between avidity scores and antibody titer. We found a strong, positive correlation between avidity scores and antibody titer ($r_s = 0.637$, $P < 0.00001$; Fig. 1a). On average, deer mice with low avidity scores ($N = 19$) had almost 12-fold lower titers than animals with high avidity scores ($N = 45$), though they were not necessarily indicative of recent infections ($W = 779$, $P < 0.00001$, excludes 7 'intermediate' avidity samples). In contrast, animals with older ($N = 60$) versus more recent ($N = 11$) infections as determined by capture history had statistically indistinguishable titers ($W = 306$, $P = 0.70$), suggesting that the correlation between titer and avidity scores is not simply driven by recent infections having lower antibody titers. Sex, site, and body-mass had no effect on titer, suggesting that these factors do not influence this relationship.

The relationship between titer and avidity scores is consistent with the pattern of misclassified animals. We observed two recent seroconversions with high avidity scores, suggestive of an older infection (Fig. 1a). We recognize that these animals may have been infected for longer than 35 days; however, it is also possible that their avidity scores were artificially elevated by high antibody titers (≥25,600; Table 1). In addition, all of the older infections with low avidity scores also had low antibody titers (≤6,400; Fig. 1a; Table 1). One animal (deer mouse 7,165) was captured in four consecutive sampling periods in 2009.

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Table 2. Comparison of analysis techniques for avidity assay performance

Performance statistic	Original	Regression with titer criteria
Sensitivity	72.6	100
Specificity	71.7	90.3
False positive rate	24.8	8.6
False negative rate	3.1	0.0
Positive predictive value	25.3	57.3
Negative predictive value	95.4	100

Its avidity scores corresponded to its recapture history until its final capture, when its titer dropped from 6,400 to 1,600 and its avidity score simultaneously dropped from 0.86 to 0.37, suggesting a declining titer of high avidity antibodies over time. Animals with older infections reverting to low avidity scores have not been previously observed (Safronetz et al. 2006). To determine if a reduction in antibody titer (such as was observed for deer mouse 7,165) could directly decrease the avidity score, we experimentally reduced antibody concentration through sample dilution and measured avidity score. The ten samples tested came from animals with older infections with titers ≥6,400 and high avidity scores at the standard 1:100 dilution. Dilution drastically decreased avidity scores for all ten samples tested ($r_s = -0.794$, $P < 0.00001$; Fig. 2).

Clearly, there is a strong association between avidity scores and the amount of antibody present, either through titer or sample dilution in the assay. We hypothesize that this association is largely a sampling artifact due to binding competition on the plate. When antibody concentration is high relative to the number of antigen molecules on the plate, high avidity antibodies will bind antigen more effectively and may selectively displace low avidity antibodies from immobilized antigen (Devey 1988). In a sample of high antibody titer, all of the binding sites may become saturated with high affinity antibodies. It may therefore be very unlikely to detect low avidity antibodies in high titer samples because they are washed off the plate before the denaturant step. In contrast, for samples of lower titer (or highly diluted samples), antigen is more readily available compared to the concentration of antibody. Low avidity antibodies will therefore bind to the plate and be affected by the denaturant treatment. Although, in general, average binding strength increases with time after infection, some low avidity antibodies persist in older infections (Werblin and Siskind 1972). These antibodies may be easily detected in older infections when overall antibody titer drops, such as was observed for deer mouse 7,165. This hypothesis may help explain the fact that apparent associations between titer and avidity scores have been reported several times in the medical literature (e.g., Hedman and Seppälä 1987; Kneitz et al. 2004), despite good evidence

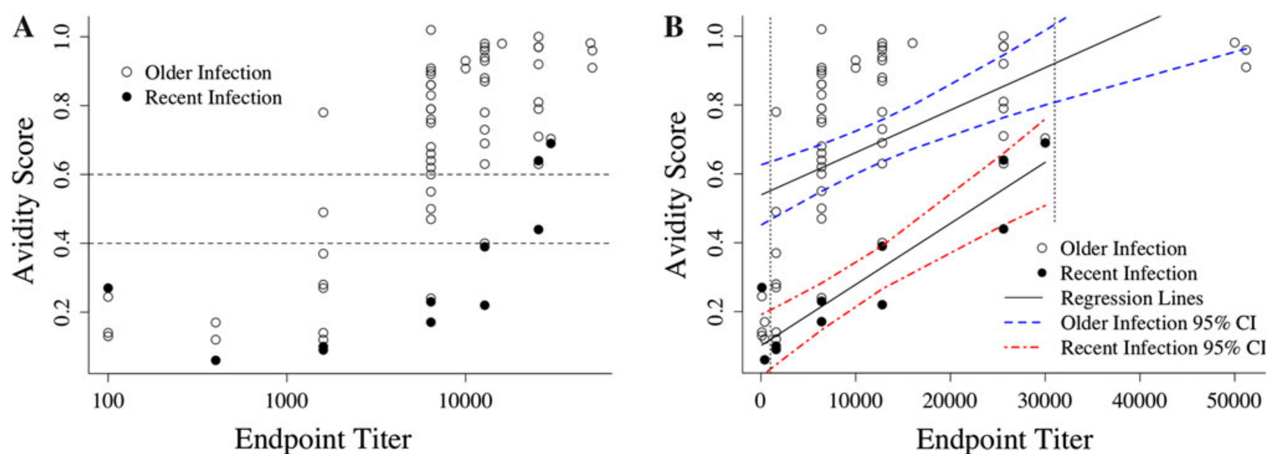

Figure 1. Titers and avidity scores for adult deer mice. **a** In the original assay, samples with avidity scores less than 0.4 were considered low avidity; samples with scores greater than 0.6 were considered high avidity. Avidity scores between the *dashed lines* at 0.4 and 0.6 are 'intermediate' and were not considered informative (Safronetz et al. 2006). **b** Linear regressions with 95% CIs were computed for older and recent infections. In this analysis, scores below the upper CI for recent infections were considered low avidity; scores above this CI were high avidity. Avidity scores where the CIs overlap are 'intermediate'. *Vertical dotted lines* represent proposed titer criteria for improving the assay's reliability in distinguishing samples from older versus recent infections. *Note*: the same infection data are shown as in (**a**) but plotted on a linear rather than logarithmic *x*-axis scale.

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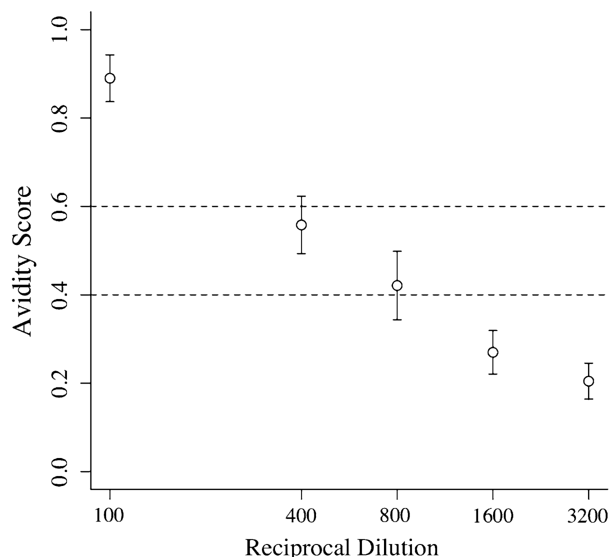

Figure 2. Effect of sample dilution on avidity scores. Average avidity score ± standard errors are plotted for ten samples with titers of ≥6,400. All samples came from animals with older infections. The *dotted lines* denote the zone of 'intermediate' avidity scores.

that the amount of antibody and its binding strength are under separate and independent regulation in the host (e.g., Kim and Siskind 1978).

With this potential mechanism in mind, we developed a regression analysis to improve the reliability of this avidity assay by controlling for titer. The recent infections in Fig. 1a consistently have the lowest avidity scores for their titer, but avidity scores increase with titer, so a linear cut-off may not be an appropriate criterion for discriminating recent infections. Instead, we computed curvilinear regression lines with 95% confidence intervals (CI) for recent and older infections (Fig. 1b). Any samples that fell below the upper CI for recent infections were considered to have a ''low'' avidity score; any samples that fell above this CI were considered to have a ''high'' avidity score. The CIs for older and recent infections overlapped for samples with titers >30,000, suggesting that they cannot be reliably classified at a 1:100 dilution. If there is competition for antigen binding sites, then low avidity antibodies are unlikely to be sampled in samples of such high titer, regardless of infection status. Avidity estimates for these samples may be improved with increased dilution, and this may be an important area for future research.

For very low titered samples (≤400), there is a very narrow margin (0.01–0.05) between avidity scores of older and recent infections (Fig. 1b). We were unable to develop a statistical method with predictive value to discriminate between samples that are so closely matched. However, by excluding seven samples with very low (≤400) and three samples with very high (>30,000) titers from the analysis, we significantly improved estimates of infection age (Table 2). The sensitivity of the assay increased to 100% (i.e., all samples from recent infections were classified as low avidity) and the specificity increased to 90.3% (i.e., most older infections were classified as high avidity). The false positive rate dropped to 8.6%, and the positive predictive value doubled to 57.3% (Table 2). Indeed, most of samples classified as ''low avidity'' by this method actually represent recent infections.

To some extent, use of this regression analysis should be informed by the research objectives. If one is hoping to infer information about individual animals or to estimate the absolute number of recent infections (e.g., for predicting human exposure risk), our data suggest that it is crucial to account for titer in the analysis of avidity data. However, use of the assay to compare the proportion of recent infections between populations (e.g., to estimate relative transmission rates between two populations) could include all samples without titer data, as long as researchers recognize that the absolute number of recent infections is likely overestimated. The bayesian positive predictive value suggests that, at least for SNV in wild deer mice, the number of recent infections is overestimated by a factor of approximately four unless one controls for antibody titer.

Our method of controlling for titer when analyzing avidity scores could easily be applied to other existing datasets. For example, Safronetz et al. (2008) reported a similar positive association between avidity scores and antibody titer in deer mice naturally infected with SNV. Our data suggest that avidity scores alone may not be able to discriminate between recent infections and older infections with lower titers. However, by controlling for titer when analyzing avidity scores, the results of the avidity analysis will likely be improved. Applying our regression analysis to the dataset of Safronetz et al. (2008) may elucidate new patterns of SNV infection in deer mice.

Avidity assays are a well-established technique in the broader biomedical literature. They are an appealing new tool for monitoring ecologically relevant pathogens and are easy to develop for any host–pathogen system with only two requirements. First, the host must produce a measurable antibody response against the pathogen, and second, sera must be available from hosts with known infection history for validation. Overall, our results indicate that antibody titer directly influences avidity scores, decreasing

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their predictive value; however, the reliability of these assays is dramatically improved after controlling for titer. In addition, there is no standard dilution among assays for testing avidity scores, but our data demonstrate that sample dilution significantly impacts avidity scores. These results highlight the importance of choosing an informative sample dilution and accounting for antibody titer when developing and validating avidity assays for new host–pathogen systems. These are particularly important considerations for pathogens that establish chronic infections because antibody titers may fluctuate during the extended course of infection.

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