Author's response to reviews

Title: Variation in dengue virus plaque reduction neutralization testing: systematic review and pooled analysis

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Response to Referees’ comments:

Referee #1:

1. “Page 6. Unfortunately, only eight vaccine studies are included in the analysis. It is not clear if vaccine studies were limited to live attenuated vaccines or a single dose of vaccine. There are certainly many human vaccine studies reported in the literature. What were the most frequent reasons for excluding so many vaccine studies? Lack of individual data? Lack of PRNT assays to all four serotypes (was this an exclusion criterion)? Were authors asked to provide individual data if only summary data was presented in their publication.”

We agree with the Referee that many important vaccine studies were excluded from this review; however, the main reason for this was because they did not present individual PRNT data. This was specifically stated in the second sentence of the final paragraph of the Background (“Here, we present a systematic review of literature reporting individual-level PRNT titers…”). We did not contact authors to acquire individual-level data, as was addressed in the first paragraph of the “Search Strategy and Selection Criteria” subsection of the Methods (“We did not attempt to contact authors to obtain unpublished PRNT titers.”)

2. “Page 6. Data abstraction. What about the use of complement in the PRNT assay? I think some labs still use complement in their PRNT and this may contribute to inter-lab differences. Also the method for plaque enumeration (direct staining vs. foci immunostain) is likely important and is probably a significant driver for the selection of virus strains since not all strains perform well by direct staining.”

We agree with the Referee’s comment about the potential effects of complement
The use of complement in the PRNT assays was not included in the analysis as this was rarely stated in the Materials and Methods sections of the included articles.

We have emphasized the importance of complement use on PRNT variation by adding a sentence to the 2nd paragraph of the Introduction:

“Examples of this variation include the addition of complement or reporting sera dilutions before or after the addition of virus.”

We have also included the lack of reporting complement use in the first paragraph of the Discussion:

“A large number of articles did not report information such as neutralization percentage, cell lines, the use of complement, and virus concentrations, making it impossible to fully adjust for these factors.”

Methods for plaque enumeration were also not collected. This is addressed in the first paragraph of the Discussion (“…heterogeneity in PRNT titers can also be induced by…plaque counting techniques such as accounting for plaque overlap…”).

3. “Page 11. In the first two paragraphs under the heading “Patterns of Reported PRNT Titers in Primary and Secondary Exposure” the authors show that primary dengue exposure leads to a principally homotypic antibody response and secondary exposure leads a more broad heterotypic response. This has been demonstrated repeatedly in the literature as early as Sabin’s work. What new information does the current study provide?”

We agree that many others have reported specific homotypic antibody responses after primary dengue virus exposure and broad heterotypic responses following secondary dengue virus exposure. Given that the pooled analysis quantifies data from a broad array of studies, we present these results to lend legitimacy to the data we collected and illustrate consistency with others’ results.

4. “Page 13. Paragraph 1. There appears to be a mistake in the strain reported to give a titer of 2.89. From Figure 3, strain CH53489 appears to be closer to 2.89 than strain 116/00.”

We appreciate this Referee’s attention to detail. Yes, strain CH53489 is the correct strain to be identified in the text. We have made this correction:

“For instance, individuals with primary DENV3 exposure had variability in unadjusted median log titers between strains ranging from 2.89 (IQR: 2.30, 3.23) for strain CH53489 to 5.56 (IQR: 5.21, 6.75) for strain 16562.”

5. “Page 14. Lines 1 and 2 and Table 3. I am confused by the data presented in
Table 3 and the conclusion that vaccination titers were 0.91 that of titers from natural exposure. If this is actually true, vaccine developers will be very happy, although I’m not sure if the majority of vaccines included in this analysis are still viable vaccine candidates. In Table 3, I interpreted the relative titers presented for the different time categories to be calculated relative to unexposed titers (“reference” designation for unexposed titers). If this is the case, then peak PRNT titers are reached 12 – 30 dpi and are 8 – 7 fold higher than unexposed (background) titers. By a year, titers have decreased to 2 – 3 fold above background. However, vaccination titers are listed as 0.8 – 0.9 compared to unexposed, which does not seem correct and is probably not what the authors intended to convey. Am I misinterpreting the data? As you can see, Table 3 needs to be improved. Probably best to divide it into at least three tables with proper headings for each column.

We understand the Referee’s confusion regarding antibody responses following vaccination being 0.8-0.9 that of individuals classified as “unexposed” in the Time categories. We have made two changes to mitigate future misunderstandings.

First, we clarified the text in the 4th paragraph of the Pooled Analysis subsection of the Methods:
“The following were also evaluated during model selection: time since exposure, neutralization percentage, cell line, virus concentration and vaccination versus natural exposure. Neutralization percentage estimates the effects of each 10% increase in neutralization percentage after adjusting for other covariates. The effect of vaccination is estimated with reference to individuals with natural exposure. Time since exposure was treated as an ordinal variable using categories described above with unexposed sera as the reference category.”

Second, we rearranged Table 3 by placing the effects of Vaccination and Neutralization percentage above the Time categories.

As Models A and B require the inclusion of the Intercept, Fixed Effects and Random Effects to calculate a PRNT titer adjusted for all other covariates, it is our opinion that splitting the table into 3 separate tables may create confusion. The Figure legend provides an example calculation to aid in model interpretation.

6. “Page 13. Middle paragraph. The authors point out that assay strains DENV2 PR-159 and DENV4 Dominica yield lower titers? Why do the authors think this is the case? What factors may be involved?”

We discuss factors that may have influenced PRNT titers in the second paragraph of the Discussion. We have altered the first sentence to emphasize that we are commenting on differences observed between strains and added a concluding sentence to specifically address this finding:
“Several additional factors may have influenced the PRNT titers collected here and differences observed between strains after adjusting for other factors in the models.”

“This may explain why DENV2 strain PR-159 and DENV4 strain Dominica/814669 produced relatively lower titers than Thai references strains.”

7. “Page 16. The authors observe that PRNT titer increases with each 10% increase in neutralization stringency. They admit that this is counterintuitive and may be influenced by their sampling. Given the data that they have collected, they should be able to test if the association between titer and PRNT stringency was influenced by the reported lower titers for PRNT90 and reported higher titers for PRNT50.”

While we agree with the Referee that testing the association between titer and PRNT neutralization level could be performed, we don’t feel this would be a substantial addition to the manuscript given its current long length.

8. “Page 17. The conclusions are brief. What new insights does this study present to the reader? Do the authors believe that vaccine-induced protection can be inferred from PRNT titers? Is the PRNT a relevant correlate of protection? I believe vaccine protection will be determined by carefully designed Phase III clinical studies. Comparison of PRNT titers from different labs will always be a challenge, even with full disclosure of the methods and assay parameters. Why do the authors believe that comparability between laboratories is actually necessary? It seems like an arduous goal without a truly compelling purpose. Why advocate for standardized strains when strain selection only accounts for 8% of the variance? What other factors are likely to contribute to the observed variability? How can these factors be elucidated.”

We added the following sentence to the beginning of the Conclusion to summarize the new insights provided by this manuscript:

“By synthesizing data from multiple sources, this analysis allows for between-laboratory and between-strain comparisons in addition to other factors that can influence PRNT titer variation.”

While it is true that it will only be ultimately possible to assess vaccine protection through carefully designed Phase III clinical studies, we do believe that PRNT titers provide useful information and remain a viable correlate of protection. We added to the Conclusion to reflect this:

“While we believe the PRNT provides a correlate of protection, the current methods do not take full advantage of quantitative results and render informal
categorization of neutralization responses. Clinical endpoints will likely be used to assess vaccine efficacy but discrepancies in protection will require more thorough assessments of neutralization titers. Systematic characterization of antigenic similarities between strains will help clarify which strains are likely to induce immunogenicity and protection against other strains, aiding in vaccine strain selection. Nevertheless, as promising vaccine candidates arise, the lack of standardized assays among diagnostic and research laboratories will make unbiased inferences about vaccine-induced protection difficult. Sources of variation have important implications for vaccine testing and comparability. Prudent study design of a candidate vaccine will ensure testing in multiple geographic locations by highly similar materials and methods, but will immunogenicity of competing manufacturers’ vaccines determined by differing materials and methods be comparable? This has direct consequences for clinical decision making and policy guidelines. Establishing methods for inter-laboratory comparisons will help unravel the complex cross-reactions that characterize dengue virus exposures.”

We have removed the use of the phrase, “reference strains” throughout the entire manuscript and introduced an emphasis on the use of reference reagents, proficiency panels and standardized algorithms to increase comparability between laboratories.

Other factors that likely contribute to the observed variability are specifically addressed in the 2nd paragraph of the Discussion. We have altered the final sentence of this paragraph to suggest possible methods for elucidating these factors:

“The use of reference strains alone will not solve this issue, but the use of reference reagents or proficiency panels would lend validity to each laboratory’s results by offering a means of quality assurance and allow each laboratory to compare “in-house” materials and methods against a standard. Alternatively, algorithms that control for protocol variations would allow laboratories to continue with their preferred materials and methods but would adjust results for comparability.”

Referee #2:

“I believe that using as reference in PRNT only prototypes strains (such as Hawaii, New Guinea C, H87, H241), those genotypes that are not circulating in all different geographical areas (countries) where studies have been conducted and serum samples collected, it is not the best way to improve dengue PRNT comparability. Related to dengue vaccine clinical trials, the objective is to evaluate population serologic response to dengue virus after immunization, thus would be better to do
the PRNT using regional reference strains once the objective is to see if the vaccine induced protection against circulating DENV serotypes in a specific region.”

We agree with the Referee’s comments regarding the use of geographically-relevant strains and mention this with regard to vaccine development in the Introduction:

“How different strains relate to one another with respect to antibody cross-neutralization has implications for the type of strains to be included in vaccine suspensions, whether a global or a region-specific vaccine should be used, as well as future research efforts after the development of a successful vaccine.”

Additionally, we have removed the use of the phrase, “reference strains” throughout the entire manuscript and introduced an emphasis on the use of reference reagents, proficiency panels and standardized algorithms to increase comparability between laboratories.

Referee #3:

“Though the question posed by the authors was well defined, it is one that has been similarly posed in recently published articles, including two referred in this manuscript (Thomas et al. 2009. Am. J. Trop. Med. Hyg., 81(5):825–833 and references 5 and 7). Likewise, the authors reached to similar conclusions reported by Thomas et al. (2009) based on actual experimental data - not on literature reports -, merely corroborating their results without providing any additional knowledge.

The statistical analysis used (log-linear hierachical models) just corroborated the conclusions obtained with other less complicated (i.e. Multivariate mixed effects linear regression models), or even empirical observations of the daily routine virology lab work with the PRNT assay.

In conclusion, this kind of manuscript/analysis is of no value what so ever.”

We strongly disagree with the Referee’s comment that “this kind of manuscript/analysis is of no value what so ever”.

First, the published articles to which this Referee refers did not pose the same scientific questions. Thomas et al. assessed the effects of covariates on PRNT results that we did not, as stated in the Introduction (“…cell type, virus passage, and the use of complement were previously identified as sources of variation and had varying effects between serotypes.”). Also mentioned in the Introduction were guidelines for standardization presented by Roehrig et al.; however, this article did not include a quantitative analysis of data. Both of these studies
influenced the design of our analyses so as to extend the work of Thomas et al. through the inclusion of additional covariates in the assessment of PRNT variability and to measure specific guidelines proposed by Roehrig et al.

Second, while experimental testing of these differences in materials and methods are important, it is also of importance to measure comparability between the results of different research groups. Systematic reviews are an ideal method for this type of comparison. Moreover, systematic reviews have intrinsic value in that they synthesize evidence from multiple sources, regardless of whether their conclusions are similar to those of other studies. Corroborating results from previous studies after the addition of other factors not only verifies knowledge but also solidifies evidence for policy recommendations and clinical decision making. We have specifically addressed this in the first sentence of the Conclusion:

“By synthesizing data from multiple sources, this analysis allows for between-laboratory and between-strain comparisons in addition to other factors that can influence PRNT titer variation.”