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Transmission-blocking activity is determined by transmission-reducing activity and number of control oocysts in *Plasmodium falciparum* standard membrane-feeding assay

Kazutoyo Miura[#1,+], **Bruce J. Swihart**[#2], **Bingbing Deng**[1], **Luwen Zhou**[1], **Thao P. Pham**[1], **Ababacar Diouf**[1], **Timothy Burton**[1], **Michael P. Fay**[2], and **Carole A. Long**[1]

[1]Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 12735 Twinbrook Parkway, Rockville, MD 20852, USA

[2]Biostatistics Research Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 5601 Fishers Lane, Rockville, MD 20852, USA

[#] These authors contributed equally to this work.

Abstract

Malaria transmission-blocking vaccines (TBVs) are potentially helpful tools for malaria eradication. The standard membrane-feeding assay (SMFA) is considered one of the “gold standard” assays for TBV development. However, lack of consensus in reporting results from SMFA has made it very challenging to compare results from different studies. Two main readouts, % inhibition in mean oocyst count per mosquito (TRA) and % inhibition in prevalence of infected mosquitoes (TBA), have been used widely.

In this study, we statistically modeled the oocyst data in SMFA using data from 105 independent feeding experiments including 9,804 mosquitoes. The model was validated using an independent data set that included 10,790 mosquitoes from 110 feeding studies. The model delineates a relationship between TRA, the mean oocyst count in the control mosquitoes (m_o-contl), and TBA. While TRA was independent from m_o-contl, TBA values changed depending on m_o-contl. Regardless of monoclonal or polyclonal antibodies tested, there were strong concordances between observed TBA and predicted TBA based on the model using m_o-contl and observed TRA.

[+]Corresponding author: Name: Kazutoyo Miura, Address: 12735 Twinbrook Parkway, Rockville, MD 20852, USA, Tel: 301-761-5071, FAX: 301-443-5778, kmiura@niaid.nih.gov.
Bruce J. Swihart (bruce.swihart@nih.gov), Bingbing Deng (bingbing.deng@nih.gov), Luwen Zhou (luwen.zhou@nih.gov), Thao P. Pham (Phuong.pham2@nih.gov), Ababacar Diouf (dioufa@niaid.nih.gov), Timothy Burton (timothy.burton@nih.gov), Michael P. Fay (mfay@niaid.nih.gov), Carole A. Long (clong@niaid.nih.gov)

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The authors declare that they have no competing interests.

Author contributions
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Simulations showed that SMFA with lower true control means had increased uncertainty in TRA estimates. The strong linkage between TBA, TRA and m_o-contl inspired creation of a standardized TBA, a model-based TBA standardized to a target control mean, which allows comparison across multiple feeds regardless of m_o-contl.

This is the first study showing that the observed TBA can be reasonably predicted by m_o-contl and the TRA of the test antibody using independent experimental data. This study indicates that TRA should be used to compare results from multiple feeds with different levels of m_o-contl. If a measure of TBA is desired, it is better to report standardized TBA rather than observed TBA. These recommendations support rational comparisons of results from different studies, thus benefiting future TBV development.

Keywords

Malaria; transmission-blocking vaccine; standard membrane-feeding assay

1. Introduction

Due to the expanded application of anti-malarial control measures, such as insecticide-treated nets, rapid diagnosis, and antimalarial drugs, the mortality of malaria has been reduced significantly in the last 15 years. However, it is estimated that 438,000 malaria related deaths, mostly due to *Plasmodium falciparum*, occurred in 2015 [1]. Multiple novel tools are likely to be required to achieve the ultimate goal of malaria eradication, and a transmission-blocking vaccine (TBV) is considered to be one of them [2-4]. TBVs are designed to induce antibodies in human hosts against sexual stage malaria antigens or to antigens found in the mosquito vector, and these antibodies should inhibit parasite development in the mosquito when they are ingested with gametocyte-stage parasites.

Several TBV candidates have reached the preclinical development stage and a few phase 1 human trials have been conducted [4,5]. To accelerate vaccine development, establishment of a robust and functional assay(s) to evaluate TBV candidates is essential [2]. There are several biological assays to determine the functionality of TBV-induced antibodies [6], and the standard membrane-feeding assay (SMFA) is considered one of the “gold standard” assays. In this assay, a mixture of cultured *P. falciparum* gametocytes and test antibodies are fed to *Anopheles* mosquitoes through a membrane-feeding apparatus, and approximately one week later the mosquitoes are dissected to enumerate oocysts in the midgut. Not only for TBV development, SMFA has also become popular for the development of drugs targeting sexual stage parasites [7-9]. However, a fundamental question relevant to this assay has not been resolved, viz., there is no consensus whether to use % inhibition in oocyst intensity (also referred to as “transmission reducing activity” or “TRA”), % inhibition in prevalence of infected mosquitoes (also called “transmission-blocking activity” or “TBA”), or both as the main readout(s) of the SMFA. The TBA readout is thought to be the best predictor of vaccine efficacy under field conditions, as a single oocyst can still generate a large number of infectious sporozoites [10]. However, one of the major differences between SMFA and natural infection is the number of oocysts per mosquito. In direct feed assays (DFA), where mosquitoes feed directly on a malaria patient’s skin [11-13], or in a study

where mosquitoes were caught in the field [14], most of the mosquitoes had less than 5-6 oocysts. On the other hand, in many SMFA assays, observed mean oocyst intensities in the control groups (m_o-contl) are much higher [10,15-17]. There is no systematic approach to judge whether TBA is still a better readout than TRA when mean oocyst intensity in the control (either m_o-contl or true mean oocysts in the control, m_t-contl) is equal to 20, 50 or 100. Inconsistency in reporting the SMFA results has made it very challenging to compare data from different studies, and has hampered application of this assay for vaccine and drug development. In addition, information on oocyst intensity and prevalence of infected mosquitoes in the control group, by which % inhibition of a test sample is calculated, are generally ignored when researchers compare the results from different assays or studies.

In this study, we first statistically modeled the SMFA using data (model-building data) from 105 membrane-feeding assays involving 9,804 mosquitoes, and then validated the model using an independent data set (validation data) included 10,790 mosquitoes from 110 feeding experiments. We utilized the SMFA model and the validation data to evaluate: 1) the linkage between TRA and TBA, and 2) the impact of control mean oocyst intensity (either m_o-contl or m_t-contl) on the error in TRA and TBA estimates.

2. Methods

2.1. Test materials

Feeding experiments were conducted with multiple monoclonal antibodies (mAb), protein G purified mouse polyclonal antibodies, and protein G purified IgGs from normal mouse, rabbit, monkey and human sera. The mAbs included 4B7 (anti-Pfs25) [18], 3E12 (anti-Pfs48/45) [19], IIC5B10 (anti-Pfs48/45) [19], and 1B3 (anti-Pfs230) [20] mAbs. The details of mouse polyclonal antibodies have been reported elsewhere [21,22], and the target antigens of those antibodies included Pfs25, Pfs48/45, Pfs230, PfHAP2 and *Anopheles gambiae* aminopeptidase N (AgAPN1). Multiple normal mouse and rabbit sera were purchased from Sigma-Aldrich (St. Louis, MO, USA), SouthernBiotech (Birmingham, AL, USA) and Invitrogen (Waltham, MA, USA). The monkey sera were obtained from Alpha Diagnostic International (San Antonio, TX, USA), and human serum from Interstate Blood Bank (Memphis, TN, USA).

2.2. SMFA

The standardized methodology for performing the SMFA has been described previously [16]. Briefly, 16-18 day old gametocyte cultures of the *P. falciparum* NF54 line (200 μl of 50% haematocrit culture adjusted to 0.15-0.2% stage V gametocytaemia) were mixed with 60 μl of a test sample, and the final mixture was immediately fed to ~50 female *Anopheles stephensi* (Nijmegen strain, three to six days old) mosquitoes through a membrane-feeding apparatus. Mosquitoes were kept for 8 days and dissected (n=~20 per “Container of Mosquitoes” (COM) for most of the cases) to enumerate the oocysts in the midgut. Throughout the paper, COM refers to a group of mosquitoes which were housed in the same container and were fed the same final mixture of gametocyte cultures and control/test antibodies. Only midguts from mosquitoes with any eggs at the time of dissection were analyzed (60-80% of mosquitoes were egg positive in general). The human serum and red

blood cells used for the gametocyte cultures and feeding experiments were purchased from Interstate Blood Bank.

2.3. Statistical analysis

Percent (%) inhibition of mean oocyst intensity (TRA) was calculated as: 100 × {1 − (mean number of oocysts in the test group)/(mean number of oocysts in the control groups)}. Similarly, the (unstandardized) % inhibition of oocyst prevalence (TBA) was evaluated as: 100 × {1− (proportion of mosquitoes with any oocysts in the test group)/(proportion of mosquitoes with any oocysts in the control group).

Details of modeling, standardization, and statistical analysis are described in the accompanying manuscript [23]. The model shows that the TBA estimand depends on m_t-contl. Briefly, the oocyst data were modeled using a zero-inflated negative binomial random effects model (ZINB model) which was similar to the method described previously [16]. In this study 9,804 mosquito data from 105 feeding experiments with 492 COMs (model-building data) were utilized to determine the best estimate of parameters in the ZINB model. The model-building data consisted of SMFA performed with normal IgGs in various species. For model validation, an independent data set including 10,790 mosquitoes from 110 feeding experiments with 541 COMs was utilized (validation data). Details of determining the 95% prediction region shown in Fig. 2 and the prediction of required number of mosquitoes in Fig. 4 are shown in the supplemental material for this paper. TBA estimates and confidence intervals shown in Fig. 5 used transformations and t-test confidence intervals [23].

The correlation between m_o-contl and TRA (or TBA) for 4B7 mAb (tested at 94 μg/ml) was determined by a Spearman Rank test. Random marginal agreement coefficients (RMACs) were utilized to determine the concordance between observed TBA and model-based TBA [24]. All statistical tests were performed in R (version 3.2.2) or Prism 6 (GraphPad Software), and p-values <0.05 were considered significant.

3. Results

3.1. Impact of mean oocysts in the control

To determine the impact of m_o-contl on % inhibition readouts, 4B7 monoclonal antibody (mAb) was tested at a fixed concentration of 94 μg/ml in 104 independent assays (Fig. 1). The median of the m_o-contl was 14.1 oocysts per mosquito (interquartile range of 7.3 to 22.8). While there was no significant impact of m_o-contl on % inhibition of mean oocyst intensity (TRA; p=0.6226 by a Spearman rank test; Fig. 1A), the % inhibition of oocyst prevalence (TBA) decreased when m_o-contl increased (Spearman coefficient = −0.563, p<0.0001, Fig. 1B). The data strongly suggest that it is difficult to evaluate the TBA readout without considering m_o-contl.

3.2. Model validation and a correlation between model-based TBA estimates and observed TBA

We recalculated the best-fit parameters of a zero-inflated negative binomial random effects model (ZINB model), which was similar to the method described previously [16], using data from 105 independent feeding experiments with 492 COMs including 9,804 mosquitoes (model-building data). The model-building data rendered the estimates of five parameters in the ZINB model; the estimate of overall control mean oocyst (log) is 2.57, the zero inflation parameter is 0.056, the standard deviation of the random effects for COM and feed are 0.2306 and 1.042, respectively, and the negative binomial dispersion parameter is 1.93. No species effect of antibodies was observed in the model fit (data not shown). For the validation of the model, an independent data set (validation data) included 10,790 mosquitoes from 110 feeding experiments and 541 COMs were utilized. As previously reported by us [16] and other groups [25,26] (regardless of control feeds or feeds with test samples), there was a strong correlation between the observed mean oocyst intensity and observed prevalence in the validation data (Fig. 2). Fig. 2 overlays the model of the true intensities and prevalence (red line) and the 95% prediction region (PR, blue lines) of the ZINB model (made by model-building data). There is a small systematic lack-of-fit in the model (more points are below and to the right of the red line than expected), but 93.5% of the validation data points were within the 95% PR boundaries of the model.

Using the ZINB model, a model-based TBA can be expressed with only 3 parameters (for details see [23] where the model-based TBA is referred to as the TBA estimand). This model-based TBA for the i^{th} sample is estimated by

$$100\left(1-\frac{1-\left(\frac{\hat{\theta}}{\hat{\rho}_i\hat{\mu}_{ci}+\hat{\theta}}\right)^{\hat{\theta}}}{1-\left(\frac{\hat{\theta}}{\hat{\mu}_{ci}+\hat{\theta}}\right)^{\hat{\theta}}}\right),$$

where $\hat{\theta}$ (=1.93) is the estimated negative binomial dispersion parameter from the model, $\hat{\rho}_i$ is the ratio of mean oocyst count in the test COM over the mean oocyst count in the control COM (so that the observed TRA for the i^{th} sample is $100\,(1-\hat{\rho}_i)$), and $\hat{\mu}_{ci}$ is the m_o-contl for the i^{th} control. We then assessed how well the observed TBA matched the model-based TBA. Using the validation data, a model-based TBA was estimated for each pair of COMs (i.e., a control and a test COM which were fed in the same experiment). Regardless of the data set analyzed, there were very strong concordances between observed TBA and model-based TBA (Fig. 3): concordance correlation (ρ^{RMAC}) = 0.92 (95% confidence interval, 95%CI, 0.89 - 0.95) for 4B7 mAb; ρ^{RMAC} = 0.90 (95%CI, 0.79 - 0.95) for other mAbs (3E12, IIC5B10, and 1B3); and ρ^{RMAC} = 0.97 (95%CI, 0.94 – 0.98) for polyclonal antibodies. The strong concordances demonstrate TRA and TBA are not independent readouts, and the observed TBA can be reasonably estimated by the model using m_o-contl and the observed TRA.

3.3. Impact of fewer mean oocysts in control on TRA estimates

The data presented strongly indicate that TBA values are difficult to compare unless all samples are tested in a single feed, or multiple feeds but with similar m_t-contl. Hence, if one wants to use the TBA readout directly to predict efficacy in a field situation, the SMFA should be performed with similar m_t-contl to that seen in the field, i.e., m_t-contl should be very low so that most of the mosquitoes have less than 5-6 oocysts. Therefore, we next estimated the impact of m_t-contl on the error in TRA estimates using the model (Fig. 4). Our simulations show that when m_t-contl is lower, many more mosquitoes are required to attain the same level of mean squared error in the TRA estimate, compared to the reference condition where 20 mosquitoes were analyzed in each COM (control and test) and m_t-contl =10. For example, when a sample with (true) TRA = 80 % is tested in a feed of m_t-contl =1, then dissection of 109 mosquitoes per COM are required to achieve the same level of mean squared error which could be obtained from 20 mosquitoes per COM with m_t-contl =10. The results indicate that SMFA assays targeting fewer m_o-contl increase the error in % inhibition estimates unless many more mosquitoes are dissected.

3.4. Standardized TBA

At present it is very challenging to achieve the m_o-contl within a certain low restricted range (e.g., the m_o-contl is always within 1-4, but not zero) and such an SMFA will likely increase the error in % inhibition estimates (Fig. 4). Therefore, we next assessed the potential of a standardized TBA using the 4B7 mAb data shown in Fig. 1. The standardized TBA values were calculated based on the observed TRA and a fixed target m_t-contl of 2, regardless of m_o-contl, using the model. In other words, the standardized TBA for the i^{th} sample just replaces the $\hat{\mu}_{ci}$ in the model-based TBA with 2. The 4B7 data were categorized based on the m_o-contl (less than 6 for “low”; between 6 and 30 for “med”; and more than 30 for “high”) and 17 data points were randomly selected for each group. As expected, unstandardized TBA values, i.e., observed TBA, were affected by m_o-contl (Fig. 5A). When the three groups were compared by one way ANOVA tests, there was a significant difference in unstandardized TBA between groups ($p<0.001$). However, there was no significant effect ($p=0.19$) of m_o-contl on standardized TBA (Fig. 5B). The results suggest that the standardized TBA should be used when SMFA data from different feeding experiments are compared.

4. Discussion

SMFA is considered as one of the “gold standard” assays, and it has been widely used for TBV development. However, different readouts, either TRA or TBA or both, have been selected to report the results, complicating comparison of results from different studies. Even within a study, if m_o-contl affects TBA and/or TRA readouts, we cannot compare data from different feeding experiments with different m_o-contl. To the best of our knowledge, this is the first study showing observed TBA can be reasonably predicted by mean oocyst intensity in the control (m_o-contl) and the TRA of the test antibody (Fig. 3) using independent experimental data. Our 4B7 mAb data from 104 assays (Fig. 1) and the strong concordance between model-based TBA (calibrated to have the target m_t-contl equal to the m_o-contl) and observed TBA (Fig. 3) convincingly indicate that TBA data cannot be

reasonably interpreted without m_o-contl; i.e., there is not one true TBA parameter, because a true TBA parameter is determined not only by the biological activity of a test antibody but also by the m_t-contl. In addition, our simulations strongly suggest that SMFA with fewer m_o-contl has a risk of increasing uncertainty in estimating % inhibition unless many mosquitoes are examined (Fig. 4, the lines in the figure were calculated using m_t-contl, not m_o-contl). Instead, our data suggest that it is better to use a standardized TBA using TRA data which are obtained from SMFA with larger m_o-contl. The standardized TBA can be utilized to compare results from different assays/studies by using any fixed target m_t-contl. Once the m_t-contl in a field site of interest is estimated, we can calculate standardized TBA using that estimate of m_t-contl from the field as the fixed target m_t-contl in the model.

Many studies from different investigators, including us, have shown that there is a strong correlation between mean oocyst intensity (either arithmetic mean or geometric mean) and prevalence of infected mosquitoes in SMFA both with *P. falciparum* and *P. berghei* parasites in different species of *Anopheles* mosquitoes (Fig. 2) [17,25,26]. We have also utilized a negative binomial model with zero inflation and random effects for COMs and feeding experiment for SMFA data, similar to models used by other investigators [25,26]. In addition, we have shown that the same model (refitting the means but using the same overdispersion and zero inflation parameter estimates) also fits with the data from the direct membrane-feeding assay (DMFA, where gametocyte parasites from infected patients are used instead of in vitro cultured gametocytes) with both *P. falciparum* and *P. vivax* (KM and Sattabongkot, personal communication, December 2015). Furthermore, Medley *et al.*, have reported a strong and a similar correlation between mean oocyst intensity and prevalence of infected mosquitoes in naturally infected *A. gambiae* and *A. funestus* mosquitoes which were collected in houses with malaria patients [25]. Similarly, Billingsley *et al.*, have also shown that a negative binomial model fits well to the observed correlation between mean oocyst intensity and prevalence in naturally infected mosquitoes if a large number of mosquitoes were dissected [14]. Therefore, we believe our ZINB model is a reasonable model to explain the oocyst data in SMFA, and it is likely to be suitable for data in DMFA and naturally infected mosquitoes. In this study, we first verified the model (Fig. 2 and 3) using independent data sets, which were not used to generate the model, then performed subsequent analysis. A more detailed mathematical discussion of model development is described in a separate manuscript [23].

It is reasonable to predict that it is more difficult to bring down the mean number of oocysts from 100 (control) to 0 (test), than from 4 to 0. A preceding study with anti-Pbs28 mAb using *P. berghei* parasites has shown that TBA level decreased as m_o-contl increased, while there was no correlation between TRA and m_o-contl [27]. Our 4B7 mAb data tested with *P. falciparum* parasites also reached the same conclusion (Fig. 1). The data from these two studies clearly show that TBA cannot be directly compared unless m_o-contl of feeds are the same or similar. However, if the linkage between the two readouts is weak, i.e., the two readouts are the indicators of different aspects of transmission-blocking activity, we need to perform SMFA with the same level of m_o-contl and always report both TRA and TBA. Therefore, we next evaluated whether they are independent readouts.

Overall, a strong concordance was observed between model-based TBA and observed TBA (Fig 3), regardless of target antigens. The result confirmed that TBA and TRA readouts are not independent, and the observed TBA is a function of m_o-contl and TRA. The lack of perfect concordance in Fig 3 is due to errors in both observed (x-axis) and model-based (y-axis) TBA. The observed TBA values have errors because of biological variability in the observed prevalence in control and test COMs. In other words, it is practically difficult to obtain the same observed TBA values when the same samples are tested in multiple feeds. The model-based TBA also has possible errors due to lack-of-fit of the model, as well as errors from the inputs into the model. For the former source of error, Fig 2 shows a small systematic lack-of-fit between observed data (validation data set) and model-based estimates (red and blue lines). However, in order to get a better fit in Fig 2, extra parameters are required (changing the 5 parameter values in the current ZINB model is not enough), and such parameters make the model more complicated. Further, our interest is not in the prevalence, but in a ratio of prevalences; i.e., TBA is 1 minus the ratio of prevalences (test sample over control sample). Therefore, the lack-of-fit of both numerator and denominator in the ratio may mostly cancel each other out. For these reasons we do not further complicate the model to achieve better fit. In terms of the inputs into the model, each of the observed TRA, the overdispersion parameter, and the m_o-contl could have errors from their true values. It is difficult to identify which error(s) dominate the discrepancy between observed TBA and model-based TBA for each data point. However, there were strong concordances overall between the two TBA.

Some investigators have been trying to control the m_o-contl levels to directly compare observed TBA values, however, as far as we are aware, no laboratory can tightly control the level of m_o-contl for every single feeding experiment. Therefore, currently the only practical method to perform a “field-like” SMFA (i.e., m_t-contl of SMFA should be very low as seen in the field) is to discard SMFA data when m_o-contl is outside a pre-defined range. This is likely to reduce the throughput of SMFA and slow the development of much needed transmission-blocking vaccines. Further, simulations under the ZINB model show that the model-based TBA (standardized to a target m_t-control) has lower mean squared error than the observed TBA (restricted to m_o-control values close to a target m_t-control) [23]. There may be considerable variability associated with the any TBA measure (model-based/standardized TBA or observed/unstandardized TBA). Thus, it is useful to express the variability for individual standardized TBA values using confidence intervals (for details see [23]). The standardized TBA also has the usual uncertainties from using an imperfect model; however, to make no adjustment at all is to essentially choose a much worse model, i.e., the TBA does not depend on m_o-contl. We have shown that the latter “model” is grossly inadequate. Sauerwein et al. specifically discussed the difference between SMFA and natural infection, i.e., m_o-contl in SMFA is usually much higher, in their recent review [28], and proposed performing SMFA with multiple levels of m_o-contl to estimate TBV efficacy in a field situation. However, the present study has shown that TRA and TBA are not independent measurements, and TBA can be reasonably estimated from TRA at a targeted m_t-control. Therefore, the results suggest that performing SMFA with multiple levels of m_o-contl is not necessary to estimate TBA in a low m_o-contl setting, although testing in multiple

feeds is always valuable, in terms of reducing errors in TRA estimates and being more model-free.

Another potential problem of a “field-like” SMFA is the larger error in % inhibition estimates. Since observed TBA depends on m_o-contl, it makes sense that the true TBA value from our model depends on the m_t-contl (see [23]). In other words, the true TBA value changes when m_t-contl changes. Therefore, the effect of m_t-contl on error in TRA estimates at each level of true TRA, not true TBA, was examined (Fig. 4). Depending on the usage of data, the tolerable levels of error in the estimates could vary. For example, when the data are used for Go and No-Go decisions in a clinical trial, one might need to modify the assays to reduce the errors, such as dissecting more than 20 mosquitoes per COM, increasing the number of COMs per feed, or testing in multiple feeds. However, the point is that our simulations clearly show that such “field-like” SMFA requires many more mosquitoes to achieve the same level of confidence which we can obtain with 20 mosquitoes per COM when m_o-contl =10. Churcher *et al.* simulated the minimum number of mosquitoes to be dissected to ensure that reported efficacy is within 10% of the true TRA efficacy [26] using their model. Their analysis also showed that many more mosquitoes were required when m_o-contl was 1 or 2 compared to an SMFA when m_o-contl was 10 or more. The two studies indicate that TRA values from a “field-like” SMFA likely have larger errors in the estimates unless many mosquitoes are dissected. Because TBA is a function of TRA and m_o-contl as shown in Fig. 3, if TRA estimates have larger errors, similarly TBA estimates have larger errors as well. There have been several attempts [29,30] to establish a higher throughput SMFA by substituting the dissection and/or oocyst counting steps at endpoint, which are labor-intensive and time-consuming procedures. These methods may be especially useful when using much larger numbers of mosquitoes than are typically used in the SMFA or when there may be limited resources available to dedicate to the labor-intensive endpoint. Regardless of how the SMFA endpoint is analyzed, the number of samples that can be tested will still be limited by the number of mosquitoes that can be produced in the insectary, potentially the volume of the gametocyte culture, and also the amount of test material available.

Taken together, to compare results from different studies (or assays) more appropriately, we recommend that: 1) TRA should be used as it allows comparison across multiple feeds over time, regardless of m_o-contl, 2) if TRA is independent from m_o-contl as it appears for all samples tested in this study, investigators should target a “higher m_t-contl” SMFA rather than a “field-like” (lower m_t-contl) SMFA to achieve smaller errors in estimates of true TRA for a given number of mosquitoes in a COM, 3) in a given feeding experiment, if m_o-contl is low, then examine more mosquitoes to increase the confidence in the TRA estimates, and 4) if a measure of TBA is desired, it is better to report standardized TBA (model-based TBA based on the observed TRA and a fixed m_t-contl, regardless of the m_o-contl) rather than observed TBA and m_o-contl. We propose these recommendations as a means to support rational comparisons of results from different studies, thus benefiting future TBV development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of abbreviations

TBV	transmission-blocking vaccine
SMFA	standard membrane-feeding assay
TRA	transmission reducing activity, % inhibition in oocyst intensity
TBA	transmission-blocking activity, % inhibition in prevalence of infected mosquitoes
DFA	direct feed assay
m_o-contl	observed mean oocyst intensity in the control group
m_t-contl	true mean oocyst intensity in the control group
ZINB model	a zero-inflated negative binomial random effects model
COM	container of mosquitoes
PR	prediction region
DMFA	direct membrane-feeding assay

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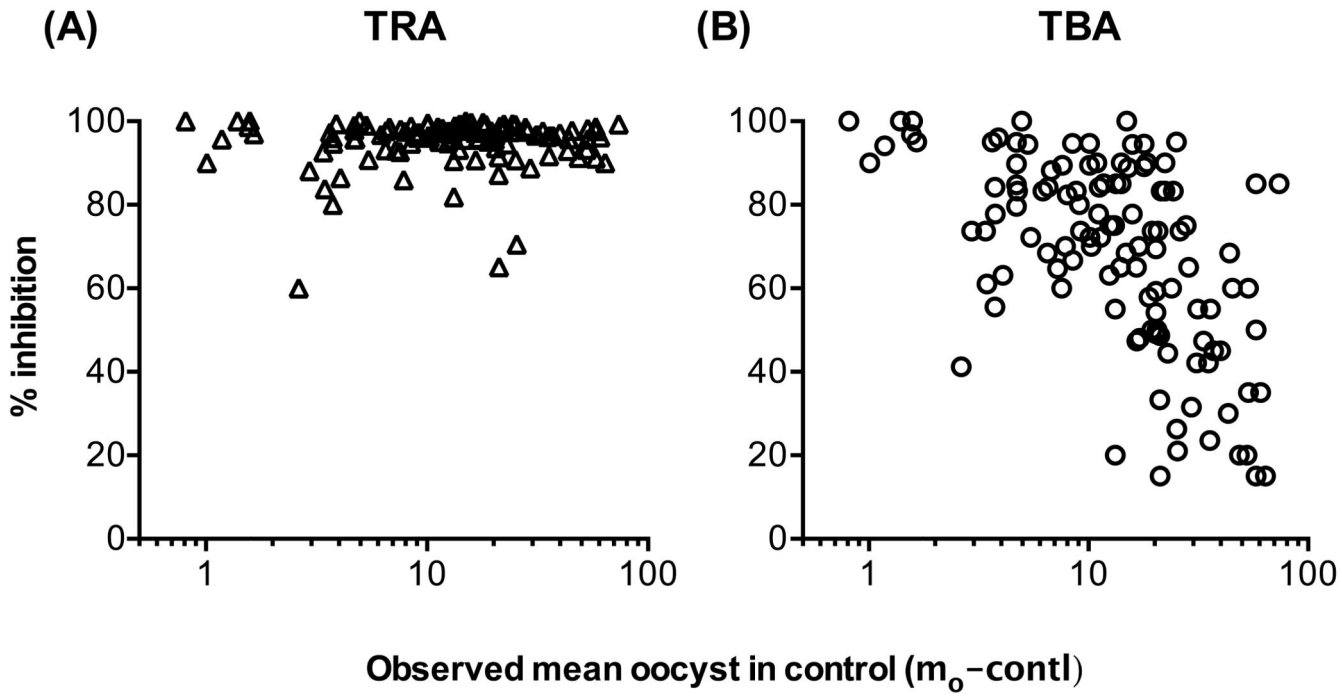

Fig. 1. SMFA with fixed concentration of 4B7 mAb

Total of 104 independent feeding experiments (124 COMs) were performed with 94 μg/ml of 4B7 mAb. The results of % inhibition of mean oocyst intensity (TRA, A) and % inhibition of oocyst prevalence (TBA, B) are shown. One data point with mean oocyst in control (m_o-contl) = 0.13 and TRA = TBA =100% is not shown in the figures.

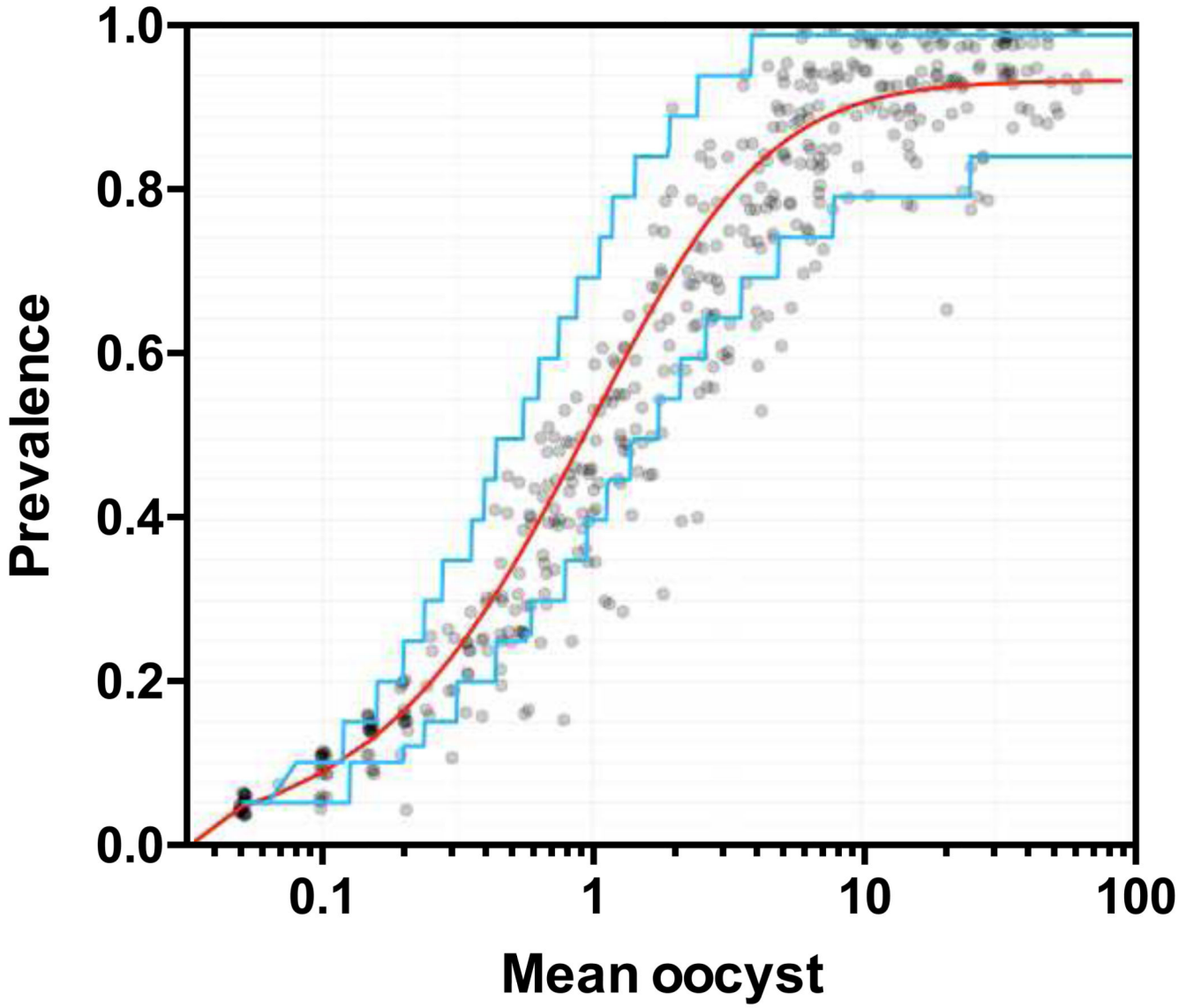

Fig. 2. Validation of the model

Independent SMFA data including 10,790 mosquitoes from 110 feeding experiments with 541 COMs were used to validate the model. The mean oocysts (x-axis in a log-scale) and prevalence (y-axis in a linear-scale) from each COM of the validation data were calculated. The data are presented with jittering and alpha blending to show overlapping points. The best-fit line (red) and the 95% prediction region (PR, blue) was estimated from the ZINB model using the model-building data (the red and blue lines are not the best-fit and the 95% PR of the validation data). The 95% PR of the model was calculated assuming 20 mosquitoes were dissected per COM (see supplement for details).

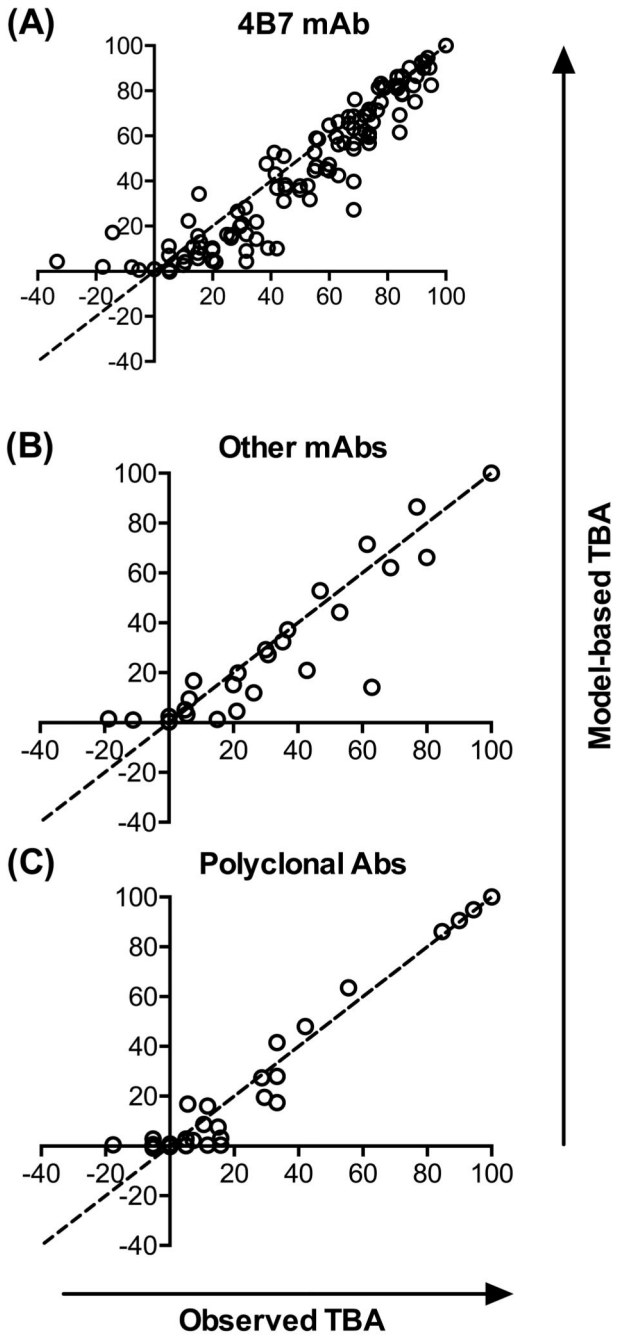

Fig. 3. Concordance between observed TBA and model-based TBA

Independent SMFA data sets (validation data) were utilized to determine whether the model could estimate TBA based on the m_o-contl and TRA of test samples. Each point represents the observed TBA (x-axis) and Model-based TBA (y-axis) with each point calibrated to the target m_t-contl that is equal to the m_o-contl of a single test sample. Data from the 4B7 mAb which targets the Pfs25 antigen (A), other mAbs targeting Pfs48/45 or Pfs230 (B) and mouse polyclonal antibodies targeting Pfs25, Pfs48/45, Pfs230, PfHAP2 or AgAPN1 (C) are shown.

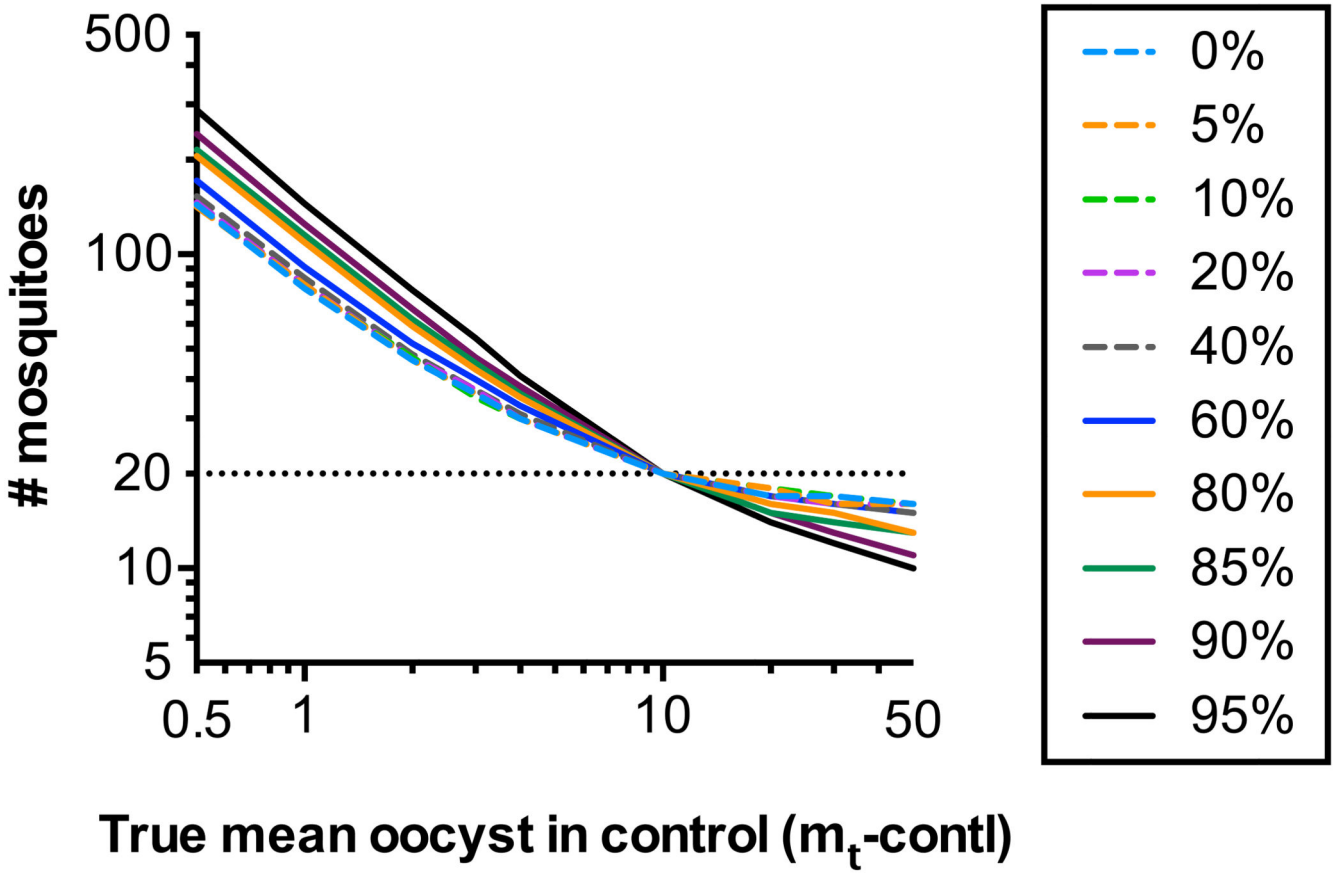

Fig. 4. Number of mosquitoes to achieve the same level of error in TRA estimates

At each level of % inhibition (TRA), the number of mosquitoes that are required to achieve the same level of mean squared error in % inhibition measurement is estimated by simulation. Different colors and symbols represent different levels of TRA (0% to 95% inhibition). The reference condition is a feed where 20 mosquitoes are analyzed in each COM (20 each for control and test) and m_t-contl =10.

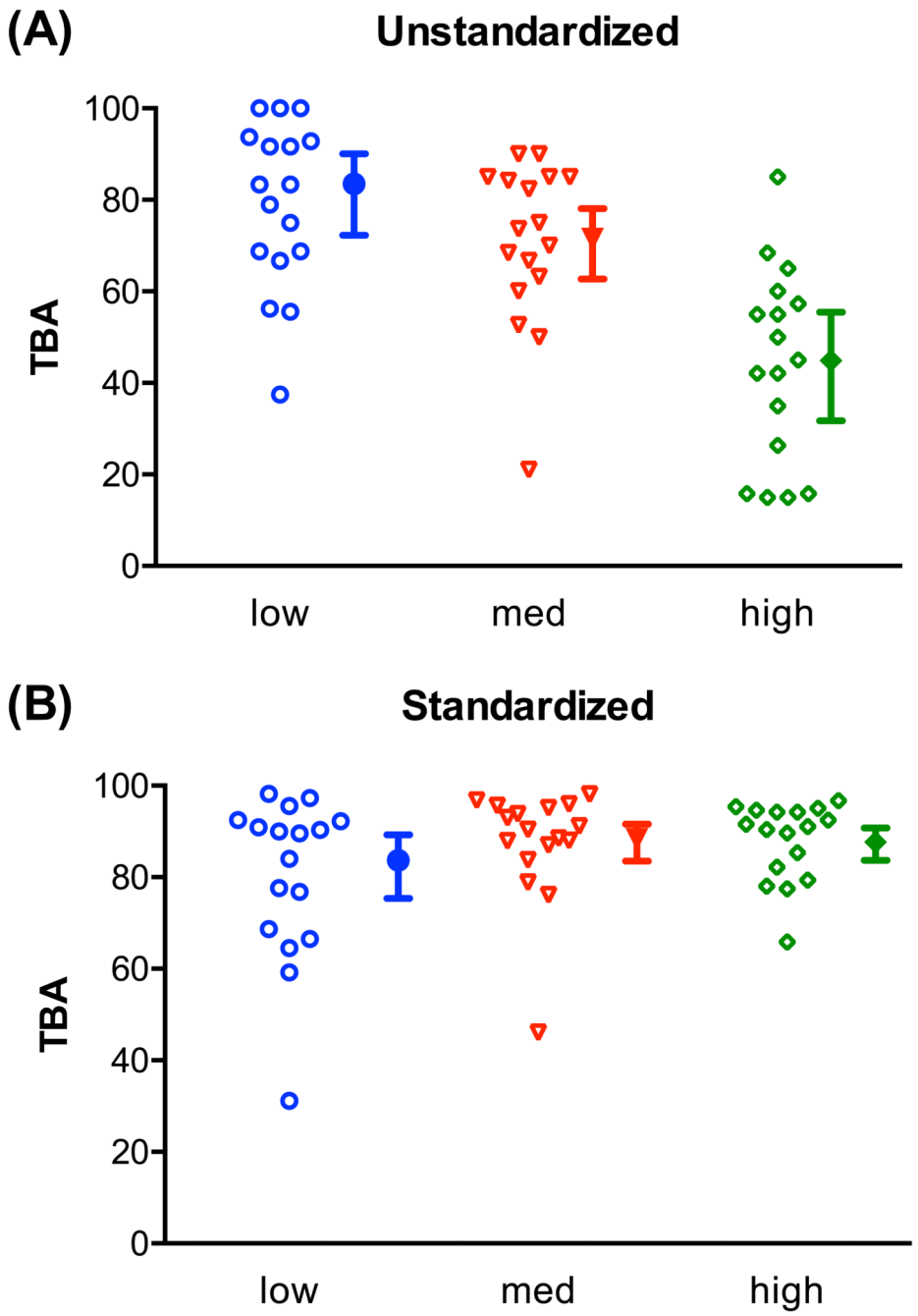

Fig. 5. Standardized TBA

From the 4B7 mAb data shown in Fig. 1, 17 data points were randomly selected in each specified m_o-contl range: m_o-contl were less than 6 for "low"; m_o-contl were between 6 and 30 for "med"; and m_o-contl more than 30 for "high". Based on the observed TRA, a standardized TBA was estimated assuming m_t-contl = 2 (regardless of m_o-contl). Unstandardized TBA (A; i.e., observed TBA) and standardized TBA (B) are shown. Both individual data, the best estimate of TBA and the associated 95% confidence intervals of each group are shown. A similar figure using the full set of 4B7 mAb data, not with

randomly selected data (n=17 per range in this figure), is shown in the accompanying manuscript [23] with 95% confidence intervals for individual points.