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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

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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 9804 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. 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.

1. Introduction

Due to the expanded application of anti-malarial control measures, such as insecticide-treated nets, rapid diagnosis, and anti-malarial 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

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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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 9804 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: $100 \times \{1 - (\text{mean number of oocysts in the test group})/(\text{mean number of oocysts in the control groups})\}$. Similarly, the (unstandardized) % inhibition of oocyst prevalence (TBA) was evaluated as: $100 \times \{1 - (\text{proportion of mosquitoes with any oocysts in the test group})/(\text{proportion of mosquitoes with any oocysts in the control group})$.

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 9804 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

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