Safety and Comparability of Controlled Human Plasmodium falciparum Infection by Mosquito Bite in Malaria-Naïve Subjects at a New Facility for Sporozoite Challenge

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Abstract

Background: Controlled human malaria infection (CHMI) studies which recapitulate mosquito-borne infection are a critical tool to identify protective vaccine and drug candidates for advancement to field trials. In partnership with the Walter Reed Army Institute of Research, the CHMI model was established at the Seattle Biomedical Research Institute’s Malaria Clinical Trials Center (MCTC). Activities and reagents at both centers were aligned to ensure comparability and continued safety of the model. To demonstrate successful implementation, CHMI was performed in six healthy malaria-naïve volunteers.

Methods: All volunteers received NF54 strain Plasmodium falciparum by the bite of five infected Anopheles stephensi mosquitoes under controlled conditions and were monitored for signs and symptoms of malaria and for parasitemia by peripheral blood smear. Subjects were treated upon diagnosis with chloroquine by directly observed therapy. Immunological (T cell and antibody) and molecular diagnostic (real-time quantitative reverse transcriptase polymerase chain reaction [qRT-PCR]) assessments were also performed.

Results: All six volunteers developed patent parasitemia and clinical malaria. No serious adverse events occurred during the study period or for six months post-infection. The mean prepatent period was 11.2 days (range 9–14 days), and geometric mean parasitemia upon diagnosis was 10.8 parasites/µL (range 2–69) by microscopy. qRT-PCR detected parasites an average of 3.7 days (range 2–4 days) earlier than drug smears. All volunteers developed antibodies to the blood-stage antigen merozoite surface protein 1 (MSP-1), which persisted up to six months. Humoral and cellular responses to pre-erythrocytic antigens circumsporozoite protein (CSP) and liver-stage antigen 1 (LSA-1) were limited.

Conclusion: The CHMI model was safe, well tolerated and characterized by consistent prepatent periods, pre-symptomatic diagnosis in 3/6 subjects and adverse event profiles as reported at established centers. The MCTC can now evaluate candidates in the increasingly diverse vaccine and drug pipeline using the CHMI model.

Trial Registration: ClinicalTrials.gov NCT01058226

Citation: Talley AK, Healy SA, Finney OC, Murphy SC, Kublin J, et al. (2014) Safety and Comparability of Controlled Human Plasmodium falciparum Infection by Mosquito Bite in Malaria-Naïve Subjects at a New Facility for Sporozoite Challenge. PLoS ONE 9(11): e109654. doi:10.1371/journal.pone.0109654

Editor: Lorenz von Seidlein, Mahidol-Oxford Tropical Medicine Research Unit, Thailand

Received July 10, 2014; Accepted August 30, 2014; Published November 18, 2014

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its Supporting Information files.

Funding: Funding for the initial MCTC infrastructure and for this clinical trial funding was provided by the PATH Malaria Vaccine Initiative (MVI) and the United States Department of Defense. MVI staff (Florence Kaltovich and Cynthia Lee) provided technical support on the chemistry, manufacturing, and control section of the IND application and the clinical operations aspects associated with this study, the latter through MVI’s clinical quality assurance consultants at The Total Approach. C. F. O. is now employed by MVI/PATH and contributed to the review of the manuscript. This work was also supported by the Intramural Research Program of National Institute of Allergy and Infectious Diseases, National Institutes of Health (S. A. H., P. E. D.), and the Global Emerging Infectious Diseases Surveillance and Response System of the United States Department of Defense (AFHSC/GEIS) under work unit number 847705.8200.2568.80016 (C. J. S.). The authors have no conflicts that affect their adherence to all PLOS ONE policies on sharing data and materials. COPYRIGHT STATEMENT: Several authors are employees of the United States Government. This work was performed as part of their official duties. Title 17 U.S.C. §105 provides that ‘Copyright protection under this title is not available for any work of the United States Government.’ Title 17 U.S.C. §101 defines a United States Government work as a work prepared by a military service member or employee of the United States Government as part of that person’s official duties. The views expressed in this article are those of the authors and do not necessarily reflect the official policy or position of the Department of the Navy, Department of Defense, nor the United States Government.

Competing Interests: The authors have declared that no competing interests exist.

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Objectives

In the absence of defined immune correlates of protection and consistently predictive animal models, controlled human malaria infections (CHMI) have become the most effective means of assessing early-stage efficacy of candidate pre-erythrocytic and erythrocytic vaccines and anti-malarial drugs. Under this model, malaria parasite-infected mosquitoes are allowed to bite human volunteers to inoculate them with Plasmodium sporozoites under controlled conditions. To date, CHMI has mostly been performed using Plasmodium falciparum-infected mosquitoes [1–8], although a few Plasmodium vivax studies have recently taken place as well [9,10]. In all trials, the subjects are monitored closely for development of patent blood-stage parasitemia and treated with standard doses of anti-malarial medications defined by the known sensitivity profile of the parasite. The parasite densities reported from CHMI studies can be used for modeling parasite growth kinetics [11,12].

CHMI studies have been conducted in the United States of America (USA) and elsewhere for decades. Reviews of the published literature indicate the model is safe, reproducible and well-tolerated by subjects participating in clinical challenge trials with P. falciparum [6,7,13]. Over the last 20 years, over 900 subjects have been experimentally infected or “challenged” with malaria at the Walter Reed Army Institute of Research (WRAIR), with an outstanding safety record and no occurrence of complicated malaria or Serious Adverse Events (SAEs). Importantly, the CHMI model plays a critical role in early phase testing of candidate interventions in order to prioritize only the most promising candidates for further product development efforts.

Despite an expanding pipeline of candidate malaria interventions in development, the number of clinical research centers capable of conducting CHMI is limited [14]. To expand the global capacity for CHMI studies, Seattle Biomedical Research Institute (Seattle BioMed) established the Malaria Clinical Trials Center (MCTC) as an innovative center for the integration of basic science and clinical research with an immediate mandate to support the development of an effective malaria vaccine. This study (ClinicalTrials.gov NCT01056226) was performed to demonstrate the successful implementation of the CHMI model at Seattle BioMed, prior to conducting efficacy trials of candidate malaria vaccines.

Methods

Objectives

The primary objective of the study was to demonstrate the safety, tolerability and infectivity of CHMI in a newly-established facility. The secondary objectives were to assess the malaria-specific immune response following CHMI in healthy malaria-naïve adults. Additionally, we sought to evaluate the detection and quantification of prepatent parasitemia by real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR).

Study Site

The study was conducted at the MCTC at Seattle BioMed in Seattle, WA. The institute also houses the Center for Mosquito Production and Malaria Infection Research (CeMPMIR), an entomology facility that was upgraded for the production of malaria sporozoites and malaria-infected mosquitoes under phase-appropriate current Good Manufacturing Practices (cGMPs) to support MCTC trials. The Clinical and Translational Research Laboratory (CTRL) was simultaneously established to support specimen processing and to perform standardized assays according to Good Clinical Laboratory Practices (GCLPs) for clinical trials.

Seattle BioMed and WRAIR established a Cooperative Research and Development Agreement to institute the CHMI model at the MCTC. WRAIR research and clinical staff from the US Military Malaria Vaccine Program served in an advisory capacity throughout preparation and conduct of the trial to ensure alignment with WRAIR practices and participant safety.

Study design

This was a prospective, open-label, single intervention study of six healthy malaria-naïve adult volunteers undergoing CHMI. All subjects were enrolled as a single cohort and followed the same study schedule (Figure 1). On the day of enrollment (Day 0), six eligible subjects underwent CHMI (“challenge”) according to standard procedures [5]. Briefly, subjects were infected with P. falciparum sporozoites by the bites of five infected Anopheles stephensi mosquitoes under controlled conditions. Volunteers were closely monitored in the post-challenge period and treated with standard oral doses of chloroquine phosphate upon diagnosis of malaria parasitemia by positive thick blood films. Periodic clinical assessments, physical examinations and laboratory monitoring were performed for evaluation of protocol-defined safety, infectivity and immunology endpoints.

Ethical conduct

This study was conducted in accordance with the International Conference of Harmonization [ICH] Good Clinical Practices (GCP) and applicable Food and Drug Administration (FDA) regulations. Based on evolving regulatory standards for human challenge studies, the P. falciparum sporozoite was considered an investigational product and for the first time, the CHMI procedures and study protocol were conducted under an investigational new drug application (IND) filed with the FDA. Prior to study initiation, the protocol and study-related documents were approved by the Western Institutional Review Board. The study was registered on ClinicalTrials.gov (Identifier NCT01058226; see http://clinicaltrials.gov/show/NCT01058226). The protocol for this trial and supporting CONSORT checklist are available as supporting information; see Checklists S1 and Protocol S1.

Study population

Subjects were recruited using IRB-approved messaging from the greater Seattle area. Subjects provided written acknowledgment of informed consent and were required to pass an assessment of understanding quiz covering key study concepts and risks related to participation. Adult males and non-pregnant females aged 18–50 years were eligible to participate in the study if they were in good general health as demonstrated by detailed medical history review, physical examination and laboratory assessment performed within 56 days of enrollment. Subjects were required to have a low risk of coronary heart disease based on the NHANES I screening criteria [15] and a normal or essentially normal electrocardiogram as read by an independent cardiologist. Laboratory assessment of eligibility included measurements of hemoglobin (Hb), white blood cells (WBC), platelets, serum creatinine, alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin and alkaline phosphatase, along with urine dipstick or full urinalysis, and screening for active infection with HIV-1/–2, hepatitis B and hepatitis C viruses. Subjects with a prior diagnosis of malaria or recent travel to a malaria-endemic area were excluded, as were those who had previously received an
of + subject received a total of five infective bites. 

investigational malaria vaccine, recent malaria chemoprophylaxis or antibiotics with anti-malarial properties. Inclusion and exclusion criteria are available in the IRB-approved protocol (Protocol S1).

Investigational product (P. falciparum sporozoites) 

Wild-type NF54 strain P. falciparum-infected A. stephensi mosquitoes for use in CHMI were reared in compliance with phase-appropriate cGMPs in the secure CeMPMIR entomology facility according to standard procedures adapted from WRAIR. The A. stephensi mosquitoes were a laboratory-reared strain originally received from WRAIR. Mosquitoes were infected with P. falciparum by standard membrane feeding methods on parasite cultures derived from the WRAIR master cell bank (MCB) containing a sufficient proportion of mature gametocytes. Prior to the CHMI, sensitivity testing of the parasite MCB to the anti-malarial drugs chloroquine, doxycycline, atovaquone and quinine was performed. The results confirmed the sensitivity of the NF-54 parasite MCB to these drugs at published values and were virtually identical to the sensitive type strain, P. falciparum 3D7, a clone of NF-54. On the day of challenge, five infected mosquitoes were placed in a screen-covered carton and allowed to feed on the subject’s forearm for five minutes. Following the feed, mosquitoes were evaluated to confirm both an adequate blood meal (as evidenced by the presence of a blood meal in the abdomen) and the presence of adequate numbers of sporozoites in their salivary glands (by individual mosquito dissections). The sporozoite load was rated microscopically according to a semi-quantitative scale: 0 (no sporozoites observed), +1 (1–10), +2 (11–100), +3 (101–1000) and +4 (>1000) [5]. Only mosquitoes with a salivary gland rating of +2 or greater were considered infective. Feeding iterations were repeated using additional mosquitoes as needed to ensure each subject received a total of five infective bites.

Post-challenge monitoring and follow up 

Subjects were closely monitored for 30–45 minutes post-challenge for acute reactogenicity and were issued a symptom diary and thermometer to record symptoms and oral temperatures daily for five days. Subjects were evaluated in clinic on Day 1 post-challenge, contacted by phone daily on Days 2-4 and evaluated again in clinic daily on Days 5–8 post-challenge. From Day 9 post-challenge, subjects were housed in a local hotel with study staff for close observation during the time they were expected to develop patent parasitemia and symptoms of clinical malaria infection. Clinical assessments conducted at each visit included symptom review, vital signs and physical examination. Peripheral blood smears were examined for detection of malaria parasites at least daily beginning on Day 5 post-infection and continuing through diagnosis and treatment. Blood samples for safety endpoint assays were collected prior to challenge, on the day of the first positive blood smear and on Days 33 and 36 post-challenge. Blood samples for immunology endpoint assays were collected at baseline, on the day of the first positive blood smear, on Days 1, 5, 35 and 56 post-challenge.

Malaria treatment decisions were based on diagnosis of patent (microscopic) parasitemia by a positive peripheral blood smear. Subjects were treated upon detection of patent parasitemia with a standard oral regimen of oral chloroquine phosphate (600 mg chloroquine base initially, followed by 300 mg chloroquine base at 6, 24 and 48 hours later) under direct observation. Subjects continued their overnight hotel stays until completion of treatment and confirmation of three consecutive daily negative blood smears, after which they were followed weekly for eight weeks. Subjects were invited to return for optional long-term immunology assessments at three and six months post-infection.

Primary safety endpoints were assessed by clinical and laboratory evaluations following the challenge and clinically significant changes from baseline status were reported as adverse events (AEs). Solicited AEs were captured on a diary card and/or by direct questioning during the 28 days post-challenge. A solicited AE was defined as a predetermined event that may reflect safety concerns related to the investigational product or events that could be reasonably expected to occur as part of the intervention. These included local and systemic signs and symptoms related to the challenge and/or clinical malaria. (Table 1). While not previously established to be related to mild malaria infection, for the purpose of ensuring safety in this initial challenge study, cardiac AEs were solicited by symptom review including chest pain. Local and systemic reactogenicity related to the challenge was collected from the day of challenge (Day 0) through Day 5 post-challenge.

Figure 1. Study flow diagram. Eighteen subjects were screened for eligibility to participate in the trial and 7 healthy volunteers were considered eligible and willing to participate. On the day of enrollment, 6 subjects were enrolled and one backup subject was discharged from the study. The six subjects underwent CHMI and completed the 56 day study. Five subjects returned for optional long term safety and immunology follow up assessments at 3 and 6 months post-challenge. doi:10.1371/journal.pone.0109654.g001
Systemic symptoms related to clinical malaria were collected from Day 5 through Day 28 post-challenge. All other AEs reported at any time during the study (Day 0 to Day 56 post-challenge) were recorded as unsolicited AEs, including any worsening or exacerbation of pre-existing conditions. All subjects were contacted by phone six months post-malaria challenge to assess for the occurrence of SAEs, chronic illnesses, or other medically significant conditions. AEs were graded by the Investigator for severity and for relationship to the investigational product. Severity was graded according to a protocol-defined toxicity grading scale, adapted from toxicity grading scales used in similar trials. AEs were coded using the Medical Dictionary for Regulatory Activities (MedDRA) and reported using System Organ Class and Preferred Term.

### Table 1. Incidence of adverse events during the 28 days following CHMI.

| Adverse Event         | N (%) | Mild | Moderate | Severe | Mean duration (days) (range) |
|-----------------------|-------|------|----------|--------|-----------------------------|
| **Solicited Local**   |       |      |          |        |                             |
| Pruritus              | 3 (50)| 2    | 1        |        | 3.3 (2–5)                   |
| Pain                  | 1 (16.7)| 1    |          | 1 (1–1)|                             |
| **Solicited Systemic**|       |      |          |        |                             |
| Abdominal Pain        | 3 (50)| 3    |          |        | 1.25 (1–2)                  |
| Arthralgia            | 4 (66.7)| 4    |          |        | 2 (1–3)                     |
| Chills                | 3 (50)| 1    | 1        | 1      | 1.67 (1–2)                  |
| Diarrhea              | 1 (16.7)| 1    |          | 1 (1–1)|                             |
| Fever                 | 4 (66.7)| 3    | 1        | 1 (1–1)|                             |
| Headache              | 5 (83.3)| 4    | 1        | 2 (1–4)|                             |
| Low Back Pain         | 2 (33.3)| 2    |          | 6.5 (4–9)|                          |
| Malaise               | 4 (66.7)| 3    | 1        | 2.25 (1–6)|                         |
| Myalgia               | 5 (83.3)| 2    | 2        | 1      | 2.8 (1–4)                   |
| Nausea                | 4 (66.7)| 1    | 3        | 1.5 (1–2)|                          |
| Vomiting              | 1 (16.7)| 0    | 1        | 1 (1–1)|                             |
| Chest Pain            | 0 (0) |      |          |        | 0 (0)                       |
| **Unsolicited**       |       |      |          |        |                             |
| Decreased Appetite    | 1 (16.7)| 1    |          | 4 (4–4)|                             |
| Dizziness             | 1 (16.7)| 1    |          | 2 (2–2)|                             |
| Insomnia              | 1 (16.7)| 1    |          | 1 (1–1)|                             |
| Cough                 | 2 (33.3)| 2    |          | 3 (3–3)|                             |
| Exertional Dyspnea    | 1 (16.7)| 1    |          | 1(1–1)|                             |

1 A single subject accounted for all severe AEs which appeared on the day of positive blood smear, and all of which decreased in severity within 24 hours of treatment.
2 Per episode (number of episodes/subjects reporting episodes).
3 Only symptoms reported from day 0 through day 5 are included.
4 Only symptoms reported from day 6 through day 28 are included.
5 Collected throughout the 56 day study.

doi:10.1371/journal.pone.0109654.t001

Malaria diagnosis

Development of patent parasitemia was monitored by microscopic evaluation of peripheral blood smears. All subjects undergoing CHMI were expected to develop patent parasitemia. Blood smears were assessed by microscopy daily from Day 5 post-infection through diagnosis and after treatment until three consecutive daily blood smears were negative. Additional blood smears were prepared at other times as needed for diagnosis in symptomatic subjects. The prepatent period was defined as the time from CHMI to microscopic diagnosis, whereas the incubation period was the time from CHMI to the onset of malaria-related symptoms.

Malaria microscopy was conducted according to standard challenge microscopy procedures adapted from WRAIR. Giemsa-stained thick blood smears were prepared in duplicate from 10 μL of blood sample spread over a 1×2 cm rectangle and examined under a high power oil immersion objective (100X) by microscopists certified to read thick blood smears for CHMI studies. Smears were considered positive if at least two unambiguous parasites per slide were identified by a study microscopist and confirmed by the lead microscopist. For asymptomatic or treated subjects, up to five passes were read for an area equivalent to 290–320 total high-power fields (hpf), allowing for detection of a parasite density of approximately three parasites/μL before declaring a blood smear negative. For symptomatic volunteers, up to 15 passes (~870–960 hpf) were scanned before declaring a smear to be negative. Quantification of microscopic parasite densities on slides from the day of diagnosis was performed by two independent microscopists who examined a minimum total of 1 μL of blood for each subject. Average parasitemia density was calculated from the number of parasites observed divided by the volume of blood examined microscopically [using conversion of volume per hpf (Table S1)].

At the conclusion of the trial, blood samples were tested using a first-generation *P. falciparum* 18S rRNA qRT-PCR assay by the
University of Washington Department of Laboratory Medicine as described [16]. Samples tested by qRT-PCR included whole blood obtained twice daily from Day 5 until the first positive blood smear as well as blood collected daily after the first positive blood smear until hospital discharge and a final sample at Day 28. Briefly, 50 μL aliquots of whole blood were preserved in 2 mL of NucliSens lysis buffer (bioMérieux) and stored at −80°C until testing. Samples were extracted on a semi-automated instrument (EasyMag, bioMérieux), and total nucleic acids were subjected to qRT-PCR to detect the P. falciparum A-type 18S rRNA and a competitive internal control [16]. The limit of quantification for the qRT-PCR assay was 20 parasites/mL of whole blood; some samples below this threshold could be classified as ‘low positive’ by melting curve analysis as described [16]. Time to detection of parasites by molecular means was calculated from day of challenge to the day that parasites were detected quantitatively by qRT-PCR at ≥20 parasites/mL.

Immune responses

Blood sampling for immunology endpoints occurred prior to challenge (baseline) and at regular intervals after challenge including Day 1 and Day 5 (corresponding to the liver stage of parasite development), the day of the first positive blood smear (corresponding to the blood stage) and post-treatment at Days 35 and 56 following challenge. In addition, subjects had the option to provide separate written consent to participate in a long-term immunology follow-up assessment with collection of serum samples on at three and six months post-challenge.

Levels of antibodies against liver- and blood-stage antigens including circumsporozoite protein (CSP), apical membrane antigen 1 (AMA-1) and the 42 kDa fragment of merozoite surface protein 1 (MSP-142) were evaluated by enzyme-linked immunosorbent assay (ELISA) as described [17]. The antigens were produced by the National Institutes of Health Malaria ELISA Laboratory. ELISA plates (Immulon 4 HBX microtiter plates, Thermo Scientific) were coated with the above antigens (10 μg per complete 96-well plate), blocked with a non-reactive blocking buffer (5% non-fat dry milk) and incubated with 100 μL of volunteer samples or malaria-positive sera (pool of 20 sera from malaria-exposed Tanzanian adults) or malaria-naïve sera (pool of 16 sera from malaria-naïve US donors) for two hours at room temperature; all sera were pre-diluted to 1:200 in 5% milk. Negative controls consisted of two wells per plate that did not receive any sera, but were otherwise subjected to all other ELISA steps. Overall ELISA results were accepted if the negative control wells had an average optical density (OD) ≤0.1 with the %CV ≤ 30% and positivity cutoffs were determined as described [17]. Individual samples were retested with dilution if ODs exceeded 3.5 or if the %CV exceeded 50% for a given sample.

Cell-mediated immune responses to CSP and liver-stage antigen 1 (LSA-1) were evaluated by interferon-gamma (IFNγ) ELISpot assay using peripheral blood mononuclear cells (PBMCs) as previously described [18]. Briefly, MultiScreen IP filter plates (Millipore) were coated overnight with mouse anti-human IFNγ capture antibody (clone 1-D1K, 10 μg/mL, Mabtech), washed and blocked. PBMCs (200,000/well) were incubated with media only (negative control), CSP- or LSA-1-derived peptide pools (1 μg/mL), cytomegalovirus (CMV) peptide controls (1 μg/mL) or phytohemagglutinin (PHA, 1 μg/mL, positive control) in 125 μL RPMI supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin for 18–22 hours at 37°C. Media only wells (no cells) were used as a plate control. After incubation, plates were washed and incubated for 2–4 hours with biotinylated mouse anti-human IFNγ antibody (clone 7-b6-1, 1 μg/mL, Mabtech) at room temperature. Plates were again washed and incubated with alkaline-phosphatase-conjugated anti-biotin antibody (1:750, Vector Labs) for 2–3 hours at room temperature. Plates were developed for 5–10 min using NBT/BCIP (nitro blue tetrazolium/5-bromo-4-chloro-3-indolylphosphate) substrate according to manufacturer instructions (Pierce). Plates were dried overnight and counted on an ImmunoSpot reader (C.T.L., Shaker Heights, OH). Sample results were accepted if the mean negative control wells spot count for a given sample was ≤20 spots/well, if the PHA control was ≥400 spots/well and if the mean spot count in media only wells (no cells) per plate was ≤6 spots/well. Positivity criteria was ≥55 spots per 10⁶ PBMCs and ≥4-fold above the mean of the negative control wells as described [18,19].

Statistics

The study was an open-label single intervention trial to demonstrate the reproducibility of CHMI at a new center. Primary and secondary analyses involved summaries and descriptive statistics for safety, efficacy (infectivity) and immunology endpoints. Comparative analyses of the primary (microscopy) and exploratory (qRT-PCR) infectivity endpoints was undertaken for the safety population and is presented as the difference in the cumulative distribution of the number of days between challenge and the first positive test (blood smear minus qRT-PCR) using a Kaplan-Meier estimator. Analyses of immunology endpoints (by ELISA and ELISpot) were performed for all subjects for whom data was available at a specific time point. Qualitative assay data analysis was performed by tabulating the frequency of positive responses for each assay at each timepoint an assessment was performed.

Results

Subject disposition

A total of 18 subjects were screened from January – March 2010 for eligibility to enroll in the study (Figure 1). Seven subjects were screen failures based on eligibility criteria, four subjects withdrew consent and six of the remaining seven eligible subjects were enrolled in the trial, including four male and two female subjects aged 19–28 years (mean 23.5 years). All six subjects were included in the safety and efficacy analyses. No subject discontinued the trial. All six subjects completed the trial through the six month follow up phone call. Additionally, five of the six subjects consented to participate in the optional blood draws for immunology samples at 3 and 6 months post-challenge.

Malaria challenge

Six subjects successfully completed the experimental infection, receiving a minimum of five infective bites. One subject inadvertently received a total of six infective bites, rather than the five defined in the protocol. This deviation was reviewed by the Medical Monitor and Safety Monitoring Committee. After reviewing safety data, both agreed that the additional infective bite did not put the subject at increased risk. As noted in Table 2, all subjects required more than one feeding iteration in order to receive a total of five infective mosquito bites. The average number of feeding iterations was 4.6 (range 2–7). In this initial study, the overall prevalence of sporozoite containing mosquitoes was 50% (Figure S1).

Safety

No SAEs were reported for any subject through Day 56 of the trial or during six months following the CHMI. The incidence of
solicited and unsolicited AEs reported as related to the malaria challenge or infection is summarized in Table 1. The mean incubation period (time to first malaria symptom) was 9.7 days (range 6–14 days; 95% CI 6.4–13.0 days; Table 2). Three of six subjects (50%) experienced at least one post-challenge local and/or systemic reactogenicity symptom between the day of challenge (Day 0) and Day 5 post-CHMI. Similarly, 50% of subjects reported at least one symptom of clinical malaria prior to the diagnosis of patent parasitemia. Two subjects (33.3%) had onset of clinical symptoms on the same day of blood smear diagnosis and one subject (16.7%) had no systemic symptoms until after diagnosis of patent parasitemia (Figure 2).

During the acute reactogenicity period (Day 0 to Day 5 post-challenge), the most common AE was pruritus localized to the site of inoculation, occurring in 3 (50%) subjects. No other AE occurred in >1 subject during this period. All subjects experienced at least one systemic AE during the 28 days post-challenge. The most frequently reported systemic AEs related to malaria were headache and myalgia each occurring in 5 (83.3%) subjects, followed by fever, malaise, nausea and arthralgia each occurring in 4 (66.7%) subjects (Table 1). Most AEs were classified as mild or moderate in severity. One subject developed severe chills, headache, malaise and myalgia on the day of first positive blood smear. The symptoms peaked in severity on the day of diagnosis and decreased in severity within 24 hours of treatment initiation. The constellation of symptoms in this subject was consistent with uncomplicated clinical malaria, and all symptoms resolved within 48–72 hours of completion of treatment.

All subjects reported at least one unsolicited AE. The majority of unsolicited AEs were mild, and most were considered unrelated to challenge procedures or malaria infection. Related events included cough, decreased appetite, dizziness, insomnia and exertional dyspnea (Table 1). Of these, only cough (2/6) was reported by more than one subject. All AEs resolved by Day 56. Subjects were followed for six months post-CHMI, and study follow-up ended in September 2010.

Table 2. Subject infectivity summary.

| Subject ID | Feeding Iterations | Time to qRT-PCR positive (days) | Prepatent period (days) | Incubation perioda (days) | Peak parasite density by methodb (parasites/mL) |
|------------|--------------------|-------------------------------|------------------------|--------------------------|-----------------------------------------------|
| A          | 4                  | 7.0                           | 11.0                   | 7                        | 9460 RTPCR 12450 BS                          |
| B          | 2                  | 7.0                           | 11.0                   | 12                       | 50170 RTPCR 53700 BS                         |
| C          | 4                  | 7.0                           | 11.0                   | 8                        | 120680 RTPCR 39000 BS                        |
| D          | 7                  | 7.0                           | 11.0                   | 11                       | 5350 RTPCR 7270 BS                           |
| E          | 4                  | 7.0                           | 9.0                    | 6                        | 3730 RTPCR 5150 BS                           |
| F          | 7                  | 10.0                          | 14.0                   | 14                       | 12880 RTPCR 2330 BS                          |
| Mean       | 4.7                | 7.5                           | 11.2                   | 9.7                      | 33712 RTPCR 19983 BS                         |
| Median     | 4                  | 7.0                           | 11                     | 9.5                      | 11170 RTPCR 9860 BS                          |
| SD         | 2.0                | 1.2                           | 1.6                    | 3.1                      | 45956 RTPCR 21206 BS                         |

1Number of rounds of mosquito feeding exposures required to achieve a total of 5 infective bites, as demonstrated by evidence of a blood meal in the mosquito abdomen and a post-feed sporozoite salivary gland score of +2 or higher. Subject D received a total of 6 infective bites while all other subjects received 5 bites.
2Number of days from CHMI to qRT-PCR-positive.
3Number of days from CHMI to peripheral blood smear-positive.
4Number of days from CHMI to symptomatic.
5BS, blood smear.

doi:10.1371/journal.pone.0109654.t002

Figure 2. Comparison of prepatent and incubation periods. Kaplan-Meier survival curve showing the percentage of subjects without patent parasitemia by blood smear (blue line) or without symptoms (dashed red line) following challenge. doi:10.1371/journal.pone.0109654.g002
Four of six subjects had no laboratory abnormalities throughout the trial. One subject had an asymptomatic mild-moderate increase in liver transaminases (ALT 160 U/L [2.5 x ULN] and AST 105 U/L [2.6 x ULN]) occurring on the same day as diagnosis of patent parasitemia (Day 11 post-challenge). The other subject experienced a mild, asymptomatic decrease in Hb from baseline (1.8 g/dL) occurring on Day 10 post-challenge that remained within the normal range. Both abnormalities resolved by Day 35.

Infectivity

All subjects undergoing CHMI developed microscopic parasitemia with a mean prepatent period of 11.2 days (range 9–14 days; SD 1.6 days) (Table 2). Four of six subjects (67%) had a prepatent period of 11 days. The earliest detection of patent parasitemia was on Day 9 post-challenge and the latest was at 14 days. As noted above, the mean incubation period was 9.7 days (range 7–14 days; SD 3.1 days). Similar to reports from other centers, there was no apparent correlation between prepatent and incubation periods (Figure 2).

Parasitemia

The geometric mean parasite density (by microscopy) at the time of microscopic diagnosis was 10.8 parasites/μL across all slides examined (range 2–69 parasites/μL; SD 21.2 parasites/μL) (Table S1). Similar to reports from other centers, there was no apparent correlation between incubation or prepatent periods and density of parasitemia at diagnosis.

Table 3. Time from anti-malarial treatment to clearance of peripheral parasitemia.

| Subject | Time to first negative blood smear (days) | Time to first negative qRT-PCR (days) |
|---------|------------------------------------------|--------------------------------------|
| A       | 2                                        | +                                    |
| B       | 2                                        | +                                    |
| C       | 1                                        | +                                    |
| D       | 2                                        | 2                                    |
| E       | 2                                        | 2                                    |
| F       | 2                                        | 4                                    |

*Data not available. Subjects were qRT-PCR positive and peripheral blood smear negative at the time of discharge from the hotel. No further daily sampling was performed after discharge until Day 28, at which point all subjects in the study were blood smear and qRT-PCR negative.

doi:10.1371/journal.pone.0109654.t003

Immunology

Humoral and cellular responses to P. falciparum antigens were analyzed for all six subjects through Day 56 and, for five of the six subjects who consented to the extension portion of the protocol, at three and six months post-challenge. Humoral responses to P. falciparum were measured by ELISA for pre-erythrocytic (CSP) and blood-stage (AMA-1 and MSP-1 42) antigens (Figure 4). Two subjects initially seronegative for CSP became seropositive, and of these two, one remained seropositive at 6 months. One subject was seropositive to CSP at baseline and remained so throughout the 6 month study period; this could be due to cross-reactive response to epitopes shared between CSP and other common antigens or remote exposure to malaria, although this was not reported by the subject. All five subjects were seronegative to MSP-1 42 at baseline.
but became seropositive on Days 35 and remained so throughout the six month study period (Figure 4). No subject was seropositive to AMA-1 at any timepoint, except for one subject who exhibited low responses on Day 0 and at three months post-CHMI, reporting an average OD of 0.12 for both timepoints (positivity cut-off was 0.11).

Only one subject showed cellular antigen-specific cell-mediated immune responses to CSP, detected on the day of the first positive blood smear (Figure 5). The CSP-specific response was negative at Day 35 and Day 56. Responses to later timepoints were therefore anticipated to be negative. As a result, PBMC samples from extension visits were not tested by ELISpot in all subjects. For LSA-1, no positive responses were detected in any subject at any timepoint (Figure 5). The mean LSA-1 response was 7.78 spot forming units (SFU)/million PBMCs (95% CI 6.08–9.47 SFU/million), similar to the negative control wells (mean 8.5 SFU/million). Based on the lack of response seen by Day 56, the LSA-1 cellular responses at three months and six months were not evaluated.

Discussion
Therapeutic and experimental malaria infection of humans has a long history, dating back nearly a century [20–22]. Chulay et al. first described the production of infected mosquitoes from in vitro cultured NF54 strain *P. falciparum* gametocytes for the experimental challenge of six healthy volunteers under controlled conditions [5]. Since that time, the CHMI mosquito bite model has continued to be an important and powerful tool in the clinical development path towards effective malaria vaccines and drugs. This study demonstrates that CHMI at the Seattle-based MCTC is safe, reproducible and well tolerated. Recent efforts at standardization of practices across centers [23] and compliance with phase-appropriate cGMP for the production of the infected mosquitoes ensures the continued safety and integrity of the CHMI model and allows for comparability in data sets across multiple centers. To this end, this is the first CHMI study conducted under an IND where the *P. falciparum* sporozoite was considered an investigational product. Similarly, efforts to align and standardize supportive diagnostic tools such as qRT-PCR [24] will further ensure comparability of data between centers.

Similar to reports from other centers, the local, systemic and laboratory AEs observed following CHMI in this study were consistent with mosquito exposure and subsequent uncomplicated clinical malaria episodes. Solicited adverse events peaked within 48 hours of blood smear diagnosis and most resolved within 72 hours. Severe symptoms (chills, headache, malaise, myalgia) observed in one subject were likewise consistent with uncomplicated malaria and resolved within 24 hours of initiating antimalarial treatment.

Consistent with the literature [13], all subjects developed signs and symptoms of malaria infection during the trial. Both the mean incubation and prepatent periods (9.7 days and 11.2 days,
respectively) were consistent with those reported in other CHMI studies (8.3 and 11.5 days) [1,3,4,6], and there appeared to be no significant relationship between incubation and prepatent periods and parasitemia upon diagnosis. Half of the subjects reported clinical symptoms of malaria prior to blood smear diagnosis, one remained asymptomatic until the first day of treatment and the remaining two had symptoms concurrent with blood smear diagnosis, which was generally consistent with the published literature [1,6]. Similarly, there was no correlation between peak parasitemia or duration of parasitemia and severity of clinical illness.

In this study, prepatent parasitemia was detected by qRT-PCR an average of 3.7 days before blood smear diagnosis, and modeling of the qRT-PCR data suggested that 28-60 (range 8-111) hepatocytes were infected per subject as previously reported [16]. In addition, there was good quantitative agreement between qRT-PCR- and microscopy-derived parasite density results on the day of patency. This study was the first to demonstrate accurate parasite detection and quantification by this qRT-PCR method. Because the 18S rRNA is biologically enriched in malaria-infected erythrocytes relative to the parent 18S rDNA by a factor of 3500–5000 [16,25], the qRT-PCR format offers robust sensitivity relative to qPCR for samples of the same volume.

In this study, mosquito infectivity was approximately 50% as assessed by the post-challenge dissection and sporozoite rating system [5], and multiple mosquito bite iterations were required to achieve a total of five infectious bites. Ultimately, 100% of subjects were infected and developed patent parasitemia. Mosquito infectivity has since been optimized, and in Seattle BioMed trials since this study, 81–91% of mosquitoes had sporozoite ratings of 2+ or higher (Jen Hume, personal communication). High sporozoite loads have also been recently noted for aseptic cGMP-grade mosquitoes used to induce infections with less than the standard five bites [3].

As expected from the limited antigenic exposure with only five to six infected mosquito bites, limited humoral and cellular immune responses to pre-erythrocytic and erythrocytic antigens were observed. Although two subjects had transient measurable antibody levels to CSP, neither maintained a detectable response at six months post-challenge. A third subject had pre-existing humoral and cellular responses to CSP that persisted at all timepoints, did not markedly increase after infection and remains unexplained; anomalous cross-reactive responses to CSP have also been reported by others [26].

Likewise, a single challenge with *P. falciparum* in our study was not sufficient to induce significant antibody response to AMA-1 as detected by our standardized assay. The peak cellular response was expected at Day 35+/−3 days, as previously reported in experimental malaria infection models [27]. In this study, cellular responses to pre-erythrocytic antigens were limited, except for two subjects with pre-existing responses to CSP that persisted at many timepoints. For CSP, there was no difference between timepoints when all subjects were analyzed together. Only one subject showed new antigen-specific responses to CSP, detected on the day of the first positive blood smear but not on Day 35 or 56. For LSA-1, no positive responses were detected in any subject at any timepoint.

In summary, this study demonstrates the successful implementation of the CHMI mosquito bite model at Seattle BioMed, thereby expanding the global capacity for conducting these important studies. Subjects were successfully infected with *P. falciparum* sporozoites, and all subjects developed patent parasitemia and were successfully treated and cured with standard doses of chloroquine. The CHMI was safe and well tolerated. The AE profile, prepatent and incubation periods were consistent with the expected clinical course and response to CHMI reported in previous malaria studies conducted at other centers worldwide. All volunteers developed antibody responses to the blood-stage antigen MSP-1, which was detectable up to six months post-challenge. Both humoral and cellular responses to pre-erythrocytic antigens CSP and LSA-1 were limited. Results from a diagnostic qRT-PCR assay correlated with blood smear density results and identified prepatent infection nearly four days before peripheral blood smears. Seattle BioMed plans to support additional CHMI studies to accelerate development of effective malaria drugs and vaccines.

### Supporting Information

**Figure S1 Cumulative mosquito sporozoite ratings for all mosquitoes used to challenge each subject.** The sporozoite load was rated microscopically according to a semi-quantitative scale: 0 (no sporozoites observed), +1 (1–10), +2 (11–100), +3 (101–1000) and +4 (>1000) [5]. Only mosquitoes with a salivary gland rating of +2 or greater were considered infective. (TIF)

**Table S1 Microscopy-based parasite density assessment.** 1Volume per 1 cm pass based on the field number for each microscope, corresponding to 290–320 hpf. Microscope/equipment numbers: Nikon Eclipse 50i, (FN 22), Nikon microscope E200, (FN20) (DOCX)

**Table S2 Comparison of microscopy and qRT-PCR-based parasite density assessments for first smear-positive sample.** 1qRT-PCR measurements as reported in [1] (converted from parasites/mL to parasites/µL). 2Quantitative agreement denoted if microscopic and qRT-PCR parasite density measurements were within 0.5 log_{10} parasites/mL of each other. (DOCX)

**Checklist S1 CONSORT checklist for MC-001.** (DOC)

**Protocol S1 IRB-approved study MC-001 protocol.** (PDF)

### Acknowledgments

The authors thank Sharon Wong-Madden for project management of this study. Jack Williams, Megan Dowler and Peter Weina provided scientific and technical advice for execution of the mosquito feeds and clinical study. 

**DISCLAIMER:** The views expressed in this article are those of the authors and do not necessarily reflect the official policy or position of the Department of the Navy, Department of Defense, nor the U.S. Government.

### Author Contributions

Conceived and designed the experiments: AKT SHK PED. Performed the experiments: AKT SAH OCF SCM CJS RW. Analyzed the data: AKT OCF SCM JK PG WCVV RW PED. Contributed reagents/materials/analysis tools: SL JW CFO DGH. Wrote the paper: AKT SCM DGH PED.
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