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Comparison Of Capillary And Venous Parasitemia Measurements In Clinical Studies Of Malaria In Ugandan Children, Adults, And Pregnant Women (cpcv)

Musleehat Hamadu
Yale University, musleehathamadu@gmail.com

Comparison of capillary and venous parasitemia measurements in clinical studies of malaria in Ugandan children, adults, and pregnant women (CPCV)

By

MUSLEEHAT HAMADU

A Thesis Presented to the Faculty of the Yale School of Public Health in Partial Fulfillment of the Requirements of the Degree of Masters of Public Health in the Department of Epidemiology of Microbial Diseases and the Global Health Concentration

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Name: MUSLEEHAT HAMADU

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Title of Study: COMPARISON OF CAPILLARY AND VENOUS PARASITEMIA MEASUREMENTS IN CLINICAL TRIALS OF MALARIA IN UGANDAN CHILDREN, ADULTS, AND PREGNANT WOMEN

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Abstract: Malaria is mesoendemic in Uganda; with an entomological inoculation rate (EIR) estimated at 562 infective bites per person year (PPY), Tororo, a rural district located in Eastern Uganda, is one of the highest transmission sites. This study leveraged participants enrolled in two NIH funded trails at the Tororo District Hospital in Tororo, Uganda in order to determine whether there is a difference in parasitemia levels and the strain dynamics of *Plasmodium falciparum* between simultaneously collected venous and capillary blood samples over the course of 42 days of follow-up.. Parasitemia levels were examined on-site via microscopy of thick and thin blood smears. Capillary and venous blood samples were blotted onto filter paper and genotyping was conducted at Yale University in order to determine whether there are differences in the strain dynamics *P.falciparum* in capillary or venous blood. In this prospective cohort study of 196 participants, a total of 587 simultaneously collected pairs of capillary and venous were examined. Parasite measurements of the capillary and venous pairs were analyzed via repeated measures of ANOVA and the means were proven to be different with a p-value of <0.001. These results add to the dearth of literature in this topic and may have implications for the protocol of clinical trials of malaria and malaria diagnosis, drug resistance testing, and drug efficacy testing in research settings.

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CHAPTER I

INTRODUCTION

Section 1: Background

The World Health Organization (WHO) Global Malaria Programme's new initiative T3: Test, Treat, Track emphasizes the scale-up of universal access to diagnostic testing, antimalarial treatment and stronger surveillance systems in malaria endemic countries [1]. The basis of T3 is malaria diagnosis; accurate diagnosis is required to determine appropriate treatment and can improve surveillance data [2]. The WHO recommends that every suspected malaria case be confirmed by microscopy or a rapid diagnostic test (RDT) before treatment [1] and uncomplicated malaria be treated with Artemisinin Combination Therapy (ACT) [2].

The diagnosis of malaria is primarily made by demonstrating the presence of parasites in erythrocytes, and the gold standard remains microscopy. Blood smears are prepared by placing one or more drops of blood upon a glass slide for thick or thin smear preparation. The blood is obtained via a capillary fingerprick or a venous blood draw. While capillary blood is best for thick and thin smears, only a small sample can be collected each time [3]. Venous blood draws, which are regarded as complicated and invasive [4], are

performed when approximately 100uL or more of blood is needed or several blood tests are conducted [3]. In community settings, blood is typically obtained by capillary finger fingerprick, while in research settings, blood may be obtained interchangeably by capillary or venous sampling. or a venous blood draw. While capillary blood is best for thick and thin smears, only a small sample can be collected each time [3]. Venous blood draws, which are regarded as complicated and invasive [4], are performed when approximately 100uL or more of blood is needed or several blood tests are conducted [3]. In community settings, blood is typically obtained by capillary fingerprick, while in research settings, blood may be obtained interchangeably by capillary or venous sampling.

Following the diagnosis of malaria, response to antimalarial therapy is judged by standard WHO guidelines for assessing antimalarial efficacy over multiple visits over 28 or more days [4, 5, 6]. During this time period, blood smears are obtained at specified intervals and examined both qualitatively and quantitatively for the presence of parasites. False positive results can lead to unnecessary treatments, contributing to extra financial costs, side effects, and the development of antimalarial drug resistance [7]. Similarly, false negative results lead to morbidity, mortality, and further transmission [5].

In addition to the use of microscopy for the diagnosis of malaria and for the determination of antimalarial efficacy, recent interest has focused upon the

accurate quantification of malaria parasitemia during the surveillance for artemisinin resistance. Evidence from Southeast Asia suggests the emergence and spread of artemisinin resistance [5,6,8]. As no molecular markers exist for the detection of artemisinin resistance, the monitoring of resistance has relied upon the determination of "parasite clearance rates" following therapy [6,9]. This is typically determined through the quantification of parasitemia over multiple blood smears, taken up to every 6-12 hours over the first several days of treatment. Importantly, in trial settings, blood may be obtained either by capillary fingerprick or venipuncture depending on the clinical setting and study protocol specifications.

In addition, capillary or venous blood samples also serve as the source of DNA for parasite genotyping in clinical trial settings. Genotyping serves to distinguish between infections caused by recrudescent strains versus new infections. It is clear that malaria strains differ in their ability to cause disease andsevere complications based, in part, on their ability to cytoadhere to capillary endothelium or other peripheral locations (brain, placental, etc...)[10,11]. It is quite possible that due to differences in cytoadherence, differences in strain dynamics may be encountered between venous and capillary samples.

The purpose of this study is to determine whether there is a difference in parasitemia levels and the presence of P.falciparum strains between simultaneously collected venous and capillary blood samples.

The proposed study presents several implications regarding malaria diagnosis,

drug resistance testing, and drug efficacy testing in research settings. While capillary and venous blood draws are used interchangeably in malarial research, there is limited evidence on the consistency of parasitemia measurements of capillary and venous blood samples [4]. In 2011 study, analysis revealed a moderately significant difference between capillary and venous blood smears in one direction ($P = 0.0059$); P. vivax parasitemia levels were higher in capillary blood than in venous blood [23]. Further research is needed to determine the consistency of venous and capillary parasitemia measurements, especially in *P. falciparum, the most prevalent strain in sub-Saharan Africa.* In study exploring *P. falciparum* population dynamics during the early phase of ACT in children with clinical malaria in Tanzania, researchers acknowledged that the definite difference in detected genotype pattern observed between two blood samples may have been due to the different blood sources: capillary and venous. To my knowledge, there are no data available comparing genotyping results for capillary and venous blood samples [24].

Section 2: Specific Aims

1) To compare parasitemia measurements over the course of 42-day follow-up for the treatment of malaria from capillary and venous whole blood in children, pregnant women and non-pregnant adults.
   Hypothesis: Due to differences in whole blood composition and sequestration of parasites in capillary compartments, significant variation in parasitemia measurements will be seen between capillary and venous whole blood as measured by microscopy.

2) To compare parasitemia measurements and parasite clearance rates over the first 72 hours following the treatment of malaria using capillary and venous measurements in children, pregnant women and non-pregnant adults.
   Hypothesis: Significant differences in simultaneous venous and capillary measurements will be present, necessitating the consistent use of measurements from the same compartment over time.

3) To compare genotyping results from capillary and venous whole blood in children, pregnant women and non-pregnant adults.
   Hypothesis: Due to differences in sequestration by parasite strains, genotyping results will differ in capillary and venous blood samples.

CHAPTER II

METHODOLOGY

Section 1: Over view

CPCV enrolled a subset of the participants from prospective NIH-funded trials investigating the pharmacokinetics (PK) and pharmacodynamics (PD) of artemether-lumefantrine (AL) in Tororo, Uganda. The CPCV study enrolled participants from 02/27/2013 – 03/04/2014 including: HIV positive and negative pregnant women, non-pregnant adults, and children. The three studies that CPCV participants were enrolled from were: an intensive PK study, a parasite clearance time study population parasite clearance study . In all of these studies, participants are enrolled at the time of presentation with uncomplicated Plasmodium falciparum malaria, and undergo venous and/or capillary collections to estimate antimalarial drug levels over 42 days.

Section 2: Study Site

The study site is a clinic of the campus of the Tororo District Hospital (TDH) located in Tororo, a rural town in south-Eastern Uganda near the Kenyan border. With an entomological inoculation rate (EIR) estimated at 562 infective bites per person year (PPY), Tororo, has one of the highest transmission sites (source). The study clinic was open 7 days a week from 8 a.m. to 5 p.m. with at least one physician or medical officer, lab technician/microscopist, and home visitor. Participants received all routine and acute medical care related to malaria during the 42-day follow-up at the study clinic. Those in need received HIV and pregnancy related care from their regular providers. However, study physicians were available if participants needed immediate care.

Section 3: Selection and Enrollment of Participants

Participants were referred from TDH or outside referral centers in Tororo District after presenting with uncomplicated *P. falciparum* malaria. Inclusion criteria for participants were: residency within 60km of the study clinic, agreement to come to clinic for all follow-up clinical and PK evaluations, and informed consent. Those considered children were between 6 months and 8 years of age and above 6kg in weight. Those that were considered adults were above 16 in age. Pregnancy was confirmed by appearance or positive pregnancy test or ultrasound. HIV status was confirmed by negative RDT test confirmed by Western Blot or HIV RNA.

Potential participants were excluded if they had a history of significant comorbidities including: active tuberculosis or other WHO stage 4 disease, current infection with non- *P. falciparum* mosquitos, consumption of any medication known to

affect CYP450 metabolism, excluding ART or prior treatment of malaria within 14 days of enrollment date, hemoglobin < 7.0 g/dL, signs or evidence of uncomplicated malaria. Potential participants that consume any of the following medication within 3 weeks prior to enrollment date were also excluded: Carbamazepine, Clarithromycin, Erythromycin (oral), Ketoconazole, Phenobarbital, Phenytoin, Rifabutin, Rifampin, Halofantrine. Grapefruit juice was avoided during the study due to its potential effects on CYP3A4.

Section 4: Sample Collection Time Points

The parent studies which CPCV participants were enrolled in followed participants for 42 day after they received ART. CPCV samples were collected during a subset of the time point from the parent study (Figure 1). Venous or capillary samples were added to pre-existing time-points within the parent study. Samples were also collected during day of failure when the participant presented to the clinic with malaria symptoms and presence of parasites was verified.

Section 5: Informed Consent

Study physician or medical officer conducted informed consent for each participant in the study clinic using the appropriate language for the adult or parent/guardians. Translaters were used when needed and the forms were available in the 5 most relavant languages in the study catchment areas: Jopadhola, Teso, Swahili, Luganda, and English. Participants has could opt out of allowing their specimen for future use. All forms were approved by approved by the Human Research Protection

Program at Yale, UCSF Committee for Human Research (UCSF CHR), Makerere University Faculty of Medicine - Research and Ethical Committee (FOM-REC) and the Uganda National Council for Science and Technology (UNCST).

Section 6: Smear Preparation and Microscopy

At each CPCV time point, a drop for a thick smear and another drop will be collected for a thin smear from both the capillary and the venous. The smears were stained with 2% Giemsa. The thick smear was used to diagnose malaria and detect parasite density, whereas the thin smear is used to determine the species of malaria parasite. The thin smear was examined only after malaria has been detected via microscopy on the thick smear. For malaria diagnosis, the number of asexual parasites per 200 white blood cells (WBCs) is counted. If there were less than 10 parasites per 200WBCs, then the count continued until 500 WBCs were counted. A slide was declared negative, if no asexual parasites were observed after counting 500 WBCs. The formulas below were used to calculate parasite density:

- # of parasites per 200 WBCs X 40 = # of asexual parasites/uL
- # of parasites per 500 WBCs X 16 = # of asexual parasites/uL

These slides will be examined were microscopy by two technicians and a third technician if there is a discrepancy. Slide preparation and microscopy techniques for CPCV were performed according to WHO guidelines [25].

Section 7: Data Collection

A study physician or medical officer recorded patient data onto standardized case record forms (CRFs). Each patient was assigned a unique identifier. Laboratory data were recorded in a log and transferred to the CRFs by a study physician or medical officer. Study coordinators routinely conducted quality and assurance of the data. Data from the CRFs were transferred into a computerized data base by specialists. This data were archived on digital tape daily; completed tapes were transported to offsite for secure storage at the Kampala Data Management Center (DMC). A subset of the data which had only CPCV time points were transferred from laboratory data log to a password-protected Excel database. To assure accuracy and security all data transfer from paper to data base underwent double entry. The two databases were merge to collect all the appropriate capillary and venous parasite density measurements for CPCV.

Section 7: Data Analysis

G*Power 3.1.6 software was used to calculate the sample size for the repeated measures of ANOVA test; with an alpha of 0.05, power of 0.95, estimated sample size was 148. These calculations were made using an effect size 0.15. The repeated measures ANOVA test via the SAS 9.3 software was used to analyze the data. These was the most appropriate tests since the researchers were comparing the parasite density of matched pairs (capillary and venous thick smears) that were matched according to an important characteristic: simultaneous time points.. The null hypothesis is that there is no difference between the means for each group (capillary versus venous). The alternative hypothesis is

that there is a difference between the means for each of the two groups (capillary versus venous).

Section 8: Blood Storage and Genotyping

Samples from capillary or venous blood were be blotted on filter paper and stored on collection cards according to WHO recommendations [4]. Thes samples were be stored in a safe place and shipped to Yale University for genotyping using a material transfer agreement and CDC importation permit. World Courier services were used for the shipment.

Researchers attempted to analyze the capillary and venous blood samples via molecular methods. DNA from blood samples obtained in Uganda were extracted via Qiagen DNA extraction kits for both venous and capillary samples. After extraction, the goal was to utlize molecular techniques to genotype strains in order to distinguish recrudescence (treatment failure due to drug resistance) and new infections. Most commonly, genotyping methods take advantage of variation in highly polymorphic genes of *P. falciparum*. Although this is achieved by PCR followed by agarose gel electrophoresis in many settings, we planned to use a more sensitive method, PCR followed by capillary electrophoresis of 3 microsatellites in the parasite to more finely distinguish strains from one another. Indeed, the World Health Organization (WHO) has recommended genotyping with capillary electrophoresis, where possible, to increase test sensitivity and discriminatory power [26]..

The importance of accurate genotyping is not only in determining drug efficacy (true drug failure versus a new infection), but also may inform us about the pathogenesis

of disease. It is known that strains of *P.falciparum* exhibit differences in adherence properties in peripheral tissues. Thus, if differences exist in the strains that are detected in simultaneous venous and capillary samples, it may suggest that certain strains are more adherent in the peripheral circulation.

CHAPTER III

RESULTS

Section 1: Data Analysis

Independent analysis was conducted over

CHAPTER IV

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