Distribution Agreement

In presenting this thesis or dissertation as a partial fulfillment of the requirements for an advanced degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis or dissertation in whole or in part in all forms of media, now or hereafter known, including display on the world wide web. I understand that I may select some access restrictions as part of the online submission of this thesis or dissertation. I retain all ownership rights to the copyright of the thesis or dissertation. I also retain the right to use in future works (such as articles or books) all or part of this thesis or dissertation.

Signature:

_____ _____

Eric Rogier Date

Short and Long Term Plasmodium Surveillance in the Low-Endemic Nation of Haiti

By

Eric Rogier

Master of Public Health
Epidemiology

_____

Philip Brachman, MD
Committee Chair

_____

Venkatachalam Udhayakumar, PhD
Committee Member

Short and Long Term Plasmodium Surveillance in the Low-Endemic Nation of Haiti

By

Eric Rogier

B.A., University of Kentucky, 2006
B.S., University of Kentucky, 2006
PhD, University of Kentucky, 2012

Thesis Committee Chair: Philip Brachman, MD

An abstract of
a thesis submitted to the Faculty of the
Rollins School of Public Health of Emory University
in partial fulfillment of the requirements for the degree of
Master of Public Health in Epidemiology, 2014

ABSTRACT

Short and Long Term Plasmodium Surveillance in the Low-Endemic Nation of Haiti

By Eric Rogier

Worldwide, deaths from malaria infection have fallen to their lowest numbers since global reporting methods were implemented in the mid-20th century. Malaria control programs, which aim to reduce human morbidity and mortality due to malaria infection in a defined geographical area, are now present in a high percentage of malaria endemic nations. In contrast, malaria elimination programs, which seek to exterminate all malaria parasites in a nation, are present in smaller numbers. Accurate malaria surveillance is required for both of these programs in order to identify regions with higher transmission intensity, disease burden, and human mortality. The nation of Haiti is thought to be a low endemic area for the malarial parasite *Plasmodium falciparum*, but inconsistent and erratic surveillance elicits many questions to the severity of malaria is on this island nation. In late 2012, a nationwide study was conducted to determine prevalence of the *P. falciparum* parasite and seropositivity among the Haitian populace. This survey involved 62 study sites which collected blood samples on filter paper for subsequent laboratory analysis. At the Centers for Disease Control and Prevention laboratories in Atlanta, GA, filter papers were processed and assayed for *P. falciparum* DNA as well as IgG antibodies against *P. falciparum* antigens. Analysis of parasitic DNA revealed a very low parasite rate in the nation of Haiti for the 2012 transmission season with 0.17% of samples positive for active malaria infection. Serological assays to determine the IgG titer in samples showed many of the individuals had been infected with the *P. falciparum* parasite at some point in their lives. Surprisingly, nationwide estimates for seropositive rates showed high rates among those in the 0-5 and 6-10 year old groups, possibly indicating a resurgence of malaria in Haiti within the past 10 years. Nationwide seropositivity curves based on the antigens MSP-1 and AMA-1 gave estimates of seroconversion rates of 0.147 and 0.275, respectively. These data show the continued persistence of malaria in Haiti and the need for consistent and nationwide surveys in the future to determine areas with the highest levels of *P. falciparum* transmission.

Short and Long Term Plasmodium Surveillance in the Low-Endemic Nation of Haiti

By

Eric Rogier

B.A., University of Kentucky, 2006
B.S., University of Kentucky, 2006
PhD, University of Kentucky, 2012

Thesis Committee Chair: Philip Brachman, MD

A thesis submitted to the Faculty of the
Rollins School of Public Health of Emory University
in partial fulfillment of the requirements for the degree of
Master of Public Health in Epidemiology, 2014

TABLE OF CONTENTS

Abbreviations Used in this Document……..1

Background…….. 2

Methods……...10

Results…….13

Discussion and Future Directions……...17

References……...23

Tables, Figures, and Figure Legends...…….....…….26

Appendices……. 40

ABBREVIATIONS USED IN THIS DOCUMENT

AMA1 – apical membrane antigen 1

CDC – Centers for Disease Control and Prevention

DBS – dried blood spot

DNA – deoxyribonucleic acid

ELISA – enzyme-linked immunosorbant assay

IgG – immunoglobulin G

LNSP – Laboratorie National de Santé Publique (Haitian national public health laboratory)

MSP – merozoite surface protein

PBS – phosphate buffered saline solution

PCR – polymerase chain reaction

PET – photon-induced electron transfer

*Pf*PR – *Plasmodium falciparum* parasite rate

PR – parasite rate

PSI – Population Services International

RDT- rapid diagnostic test

SDE – sélectionner par département (study location within department)

SOP – standard operating procedure

TRaC – tracking results continuously survey

BACKGROUND

Throughout human history, malaria parasites have been a significant burden to our species. In some areas of the globe, this affliction has been so great as to affect entire civilizations and even to the extent of modifying the human genome. Blood disorders including sickle-cell disease, thalassemias, and others are thought to have originated in malaria-endemic areas and confer a degree of protection against severe malaria, leading to the hypothesis that in the past these disorders were actually selected for within human populations (1,11). Still today, nearly one million fatalities are attributed to this disease every year with nearly all of the victims being children under the age of five (2). Over the past century, tremendous and successful efforts to stymie the deadly impact of malaria have provided much optimism that this disease can be eliminated within certain areas, and ultimately eradicated worldwide.

Humans are the definitive host and reservoir for the human malarial parasites, and global migration of the parasite is due to migration of people, whether it manifest in the form of immigration or temporary travel. It is currently thought that malaria originated in Africa, by mutations that allowed simian malarias to jump to African peoples around 50-100,000 years ago (3). Before European explorers sailed across the Atlantic Ocean, malaria did not exist in the New World (4). This, along with other infectious maladies carried in the bodies of the explorers, quickly lead to enormous amounts of morbidity and mortality for the Native Americans and has been a poignant example of how quickly malaria can spread in a naïve population. It is known today that human malaria can be caused by five species of apicomplexan parasites, all within the genus *Plasmodium*. The disease also affects other species of animals – from birds to non-human primates – all by infection with a species of *Plasmodium*. Interestingly, human malaria does not segregate itself to a distinct portion of the *Plasmodium* phylogenetic tree, and it has been found that some human parasites are more closely related to avian malaria than to other species of human-infecting parasites (5). In fact, the parasite *Plasmodium falciparum* has been estimated to be separated from the other parasite species by 100 million years of evolution (5). The parasite *P.*

falciparum is known to be the most virulent of the human malarias, and has a widespread global distribution – being endemic to every continent with the exception of Australia and Antarctica (Ref 6, Figure 1). With Australia's recent certification of malaria non-endemicity, Europe is also hopeful that the final vestiges of the malarial parasite can be eliminated within its borders in the next few years.

The course of infection with the *P. falciparum* parasite is a very complex process, and has only begun to be well understood in the latter half of the twentieth century. Though it has been shown that persons can be infected with *P. falciparum* through blood transfusion (7,8), by far the most common acquisition of infection occurs through the bite of the infected *Anopheles* mosquito. Only certain *Anopheles* are able to transmit *P. falciparum* with the African *A.gambiae* being currently known as the best vector (9). Before biting the human host, the midgut of the mosquito has already been the site of sexual reproduction for the parasite (10), which allows for greatly-enhanced genetic diversity for the pathogen and provides an evolutionary advantage by allowing many diverse genotypes to arise from the mating of two parasites. These diverse genotypes have the increased likelihood of responding to a multitude of environmental conditions and continuing to perpetuate. Following division within the mosquito midgut, parasites travel to the salivary glands, where they are released into the human host when the mosquito takes its next blood meal. These parasites, termed sporozoites, quickly home to the host liver where they undergo multiple divisions before rupturing into the bloodstream (1,10). These merozoites can now infect erythrocytes, divide, and then re-infect other erythrocytes after cell rupture, leading to a cyclic infection that takes 48 hours for *P. falciparum* to complete. During this cyclic process, involving the erythrocytes, some parasites take on a sexual form which is able to be taken up by a feeding mosquito. A simplified diagram of the entire *P. falciparum* life cycle within the human host can be found in Figure 2 (1).

Fortunately, as humans gain more infections with *P. falciparum*, they are able to mount a robust immune response to the malarial parasite. Human mortality due to malaria is highest in

areas with: a high percentage of inhabitants of young age, a high transmission of *P. falciparum*, and malnourishment within the population. These three factors all come into play in Sub-Saharan Africa, where malaria mortality under five years of age accounts for 75% of all malaria deaths worldwide (2). Though this situation is quite sobering, it has been shown that immunity to severe complications associated with malaria can be acquired after only a few infectious episodes (12). After age 5 years of age, those living in malaria-endemic areas have a very low chance of developing severe complications upon subsequent infection. Those coming into an endemic area (such as travelers from a developed country) are quite susceptible to severe infection, and prophylactic medications are highly recommended for these people. The best understood immune response to *P. falciparum* infection is the humoral response which is maintained in the host by populations of memory B cells. Since *P. falciparum* hides itself within erythrocytes most of the infection's duration, it is generally thought that multiple infections allow sufficient interactions with host immune cells and are needed before efficacious IgG antibodies are able to be produced and memory B cell populations established (13). However, once established, B cell populations specific to some of malaria's most immunogenic antigens have been found in persons exposed only one time over a decade ago (14). This is quite helpful in establishing the history of infection not only for an individual but also a defined geographic area. The dosage and exposure to *P. falciparum* required to produce a certain quantity of IgG antibodies in an individual is nebulous, but it is well known that persons in high-endemic areas will continually have a high titer, and persons with only one past exposure will generally always have detectable (albeit low) titers of antibodies (13). When assessing the past prevalence of malaria in a population, two pieces of information will provide a wealth of information when used in conjunction: the person's age and the presence of anti-malarial antibodies. This information can be looked at in a dichotomous fashion (positive/negative) and scatter plots created showing positivity with age. Using the dependable assumption of seropositivity after exposure to malarial parasites (or, at most, two exposures (12)), persons will remain seropositive for decades, if not the rest of their

life. In high-endemic areas such as central and western Africa, the proportion of the population that is seropositive will be near 100% around an age of 6-10 years (17). However, in a low-endemic area where the population is not continually exposed to malarial parasites, seropositivity may reach an asymptote of around 20% at the study population age of 20-30 years (18). An even more interesting phenomenon occurs when a successful regional malaria elimination effort has taken place in the past and malaria has become dramatically less endemic in a short period of time. In this instance, the seropositivity curve takes on two segments, the before-intervention and after-intervention curves. All persons born after the successful intervention will show a lower rate of acquired seropositivity (a less-steep curve with lower asymptote), while person born before the intervention will have the high rate of acquired seropositivity as indicated by the high asymptote (19). Examples of seropositivity curves for the three scenarios above are given in Figure 3. If a true break in the seropositivity curve exists, a researcher can even retrospectively evaluate events that would have occurred around the time of the break leading to the prolonged lower malaria endemicity.

As with many diseases, not all persons infected with parasites show symptoms of a disease. This reality alludes to the "iceberg of infection" and the difficulty of elimination efforts even when humans are the only reservoir for the infectious agent. While these asymptomatic persons would still mount an antibody response, they are still potentially infectious in the sense that mosquitoes would be able to leech parasites from their blood to transmit to another person (21). In many malaria-endemic countries, all blood donors are screened for malarial parasites, since receiving a contaminated blood transfusion would almost certainly be fatal due to the high initial parasitemia (8, 22). Though blood donors who would test positive would be informed of their malaria carriage, a more active mode of disease intervention is known as the "screen and treat" strategy. In this approach, all persons within a defined population are screened for malaria infection by microscopy or rapid diagnostic test (RDT) and treated with the appropriate anti-malarial drug if infected. Both microscopy and RDT methods for detection of malaria infection

are quite effective when the parasite load is high in the blood, but these methods become very unreliable when parasite load dips below 100 parasites/uL blood. Since the majority of the infectious period will occur during submicroscopic (and sub-RDT) periods, reliably finding those who harbor a low infection requires more sensitive methods (23). Figure 4 shows the importance of being able to detect submicroscopic infections if malarial elimination is to be achieved. Though not currently practical for bedside diagnostic purposes, a much more sensitive assay is available that amplifies parasitic DNA by polymerase chain reaction (PCR). In a recent meta-analysis of 106 studies, PCR was found to be more sensitive than microscopy for every study (23). One of the major downfalls to the PCR assay is the requirement of many of the luxuries of a laboratory such as a freezer, sterile spaces, and trained technicians which may be unavailable in developing countries. In addition, the reagents required for running PCR assays are expensive relative to microscopy and RDTs. Although not useful for case detection in the field, PCR offers many advantages for conducting surveillance studies. Standard limits of detection hover around 1 parasite/uL blood with some assays achieving detection in the range of 10^{-3} to 10^{-2} p/uL (24,25). Many varieties of PCR assays exist, allowing the quantitative detection of the 5 different malarial parasites, as well as further genetic analysis such as genotyping and recognition of antimicrobial resistance haplotypes. The types of samples that can be analyzed by PCR are numerous, since detection limits are so low and considerations for DNA integrity do not have to be stringent. In surveillance studies, a common methodology used is blood spotted onto a filter paper. This type of sample only requires a finger prick to gain the sample, and can be stored at ambient temperature for long periods of time without catastrophic degradation of DNA. As is important in surveillance studies, a large number of samples can be obtained in a short amount of time, and the persons collecting samples need minimal training.

Since malarial parasites can only be transmitted in areas indigenous to the *Anopheles* mosquito, vector control efforts during the era of European colonialism helped to greatly reduce the annual cases and deaths brought on by complications from malaria. Even today, vector

control efforts are thought to be one of the most effective ways to prevent cases of human malaria (10). Colonial efforts to reduce the prevalence of malaria almost always included the use of the pesticide DTT. Efforts in many nations made considerable success, even eliminating malaria in large regions such as Australia, the United States, and many Caribbean islands. However, the mid-20th century ban on DDT in developing countries showed the rapid re-emergence of malaria as mosquito populations rebounded (26). It was soon found that the burden of malaria was too great, and DDT was reintroduced to some nations. After the initial elimination of malaria from some nations a good distance away from the equator, optimism abounded that soon the disease would be wiped from the earth. As with so many infectious diseases, this notion was quickly dispelled by the enormous efforts needed just to prevent deaths, much less eliminate parasites. Great strides have been made in areas far from the equator, and of the 178 countries with malaria in the first half of the 20th century, 79 countries eliminated the disease by 2010 (27). Of these remaining 99 countries, 67 are implementing control programs while the other 32 are working towards elimination (27). A nation with a control program is very likely in an area of high transmission and/or financially dependent on other nations for funding. The concept of elimination is desirable for a nation since funding once used for malaria control can be diverted to other activities. Elimination is not an inflexible term, but certainly includes the interruption of endemic malaria but allows for the possibility of reintroduction. The United States has technically eliminated malaria, but has also seen outbreaks from introduced parasites in the past 50 years (28, 29). Ultimately, the worldwide goal is malaria eradication, where the global case count reaches zero and neither clinical nor subclinical infections exist. The fact that no known zoonotic source exists for *P. falciparum* and many tools area being implemented to combat this pathogen leads to optimism of eventual eradication (31). Malaria elimination is a true team effort with international involvement from organizations such as the World Health Organization, Global Fund, President's Malaria Initiative, and the CDC. Involvement with national ministries of health and the populace is also crucial to work towards the same goal.

Islands serve as attractive geographical areas for malaria elimination due to their non-contiguous borders and smaller size. Additionally, reintroduction of the parasite to the island would require an infected human (or, less likely, an infected mosquito), and screening strategies can greatly reduce this incidence from taking place. Another benefit to elimination efforts in island nations is the ability to engage a large percentage, or even all, of the local population (30). Every island in the Caribbean Sea has been able to eliminate malaria with the exception of Hispaniola. This island is split almost evenly between two nations: Haiti and the Dominican Republic. Elimination efforts have been robust in the Dominican Republic, and the country is now estimated to have less than 3,000 cases per year (31). In contrast, Haiti is only considered to be in the process of malaria control (2), and shows somewhere between 100,000 and 200,000 cases per year (31). The precise number and geographical distribution are both very vague due to the paucity of passive and active surveillance efforts. Figure 5 provides a rough prediction of the clinical burden of malaria by geographical area in Haiti, but is a loose patchwork of data from past years from many organizations. The only comprehensive and reliable malaria survey preformed in Haiti took place in 2005 when 35% of the population of nearly ten million was screened by microscopy (2).

Malaria elimination in Haiti is of high importance, not only to the inhabitants of the island, but also directly to the United States. Figure 6 shows the number of imported *P. falciparum* cases to the United States by persons returning from Haiti. Due to increased temporary travel to Haiti by family and aid workers in the wake of the 2010 earthquake, imported malaria cases rose to almost 200 in the year 2010 – rivaling America's numbers of almost all imported cases from Africa (32). If not properly detected and treated, each of these cases provides the potential for an outbreak if the persons are bitten by a mosquito. The push for current elimination efforts is also fueled by the inevitable encroachment of chloroquine resistance which is already widespread in almost all other *P. falciparum* endemic areas. No consistent data has shown chloroquine resistance as being present in Haiti, but *P. falciparum*'s development of

this phenotype is a constant concern for public health officials. Since practically all malaria in Haiti is *P. falciparum*, all parasites are susceptible to inexpensive chloroquine, and the vast majority of transmission occurs from October to December, elimination will only need to take multiple groups working together and commitment from the Haitian government (33). Accurate surveillance will be necessary not only for elimination of malaria from Haiti, but also to help prevent subsequent reintroduction from imported cases. Following successful elimination, imported cases of malaria will still be a concern for Haiti, since the mosquito vector will still be present in the region. Along with a diagnose-and-treat strategy, efficient and rapid surveillance will be able to recognize cluster outbreaks in the future and can lead public health officials to the correct geographical areas to quell these outbreaks before *P. falciparum* reestablishes endemicity.

METHODS

Study Design. In late 2012, a study entitled "Tracking Results Continuously (TRaC) Protocole de Recherche: Étude comportementale et épidémiologique sur le Paludisme en Haïti, Passage 2" was conducted by the Haitian affiliate of Population Services International (PSI, headquarters in Washington DC). As per standard protocol, the TRaC survey collected data among the Haitian target population through household surveys where each person in the house was requested to provide a blood sample. This study, a nationally representative, household survey, was conducted in December 2012 without further subject contact. Consent was obtained to collect from subjects, drops of blood on filter paper to test for presence of malarial DNA and anti-malarial antibodies by CDC laboratory analysis. From each individual, peripheral blood drops were collected by lancet puncture of the index finger, and spotting onto filter paper with one blood drop per filter paper spot. PSI did not send CDC specimens containing personal identifiers and CDC involvement does not constitute "engagement in human subject research" and does not require IRB review or approval.

Processing and Splitting of Dried Blood Spot Samples. Once collected in the field, samples were shipped to the Haitian National Public Health Laboratory in Port-au-Prince (LNSP) at ambient temperature. Upon arrival at LNSP, samples were stored at 4^{o}C until further processing. Since multiple spots are collected for each individual and both CDC and LNSP utilized samples for analysis, samples on a Whatman 903 Booklets or Whatman 1 circular filter paper were split between the two research laboratories (Appendix A). After shipping of samples to the CDC laboratories in Atlanta, GA, samples were stored at 4^{o}C until DNA or protein elutions were processed within the CDC laboratories.

DNA Elution from Dried Blood Spots. Samples for elution were allowed to return to room temperature. The protocol for DNA elution from spots was based on the standard procedure

outlined by the Qiagen DNA mini kit (Qiagen, Hilden, Germany). Due to the desire to get a higher amount of blood per PCR reaction and to conform to upcoming international standards, the Qiagen protocol was modified to include the entire blood spot in the DNA isolation and elution. This modified protocol can be found in Appendix B.

Polymerase Chain Reaction for *P. falciparum* DNA Detection. Initial screening for presence of any DNA within the genus *Plasmodium* was accomplished using the PET-PCR methodology initially developed by Lucchi (16) with the HEX (6-carboxy-2 ,4,4 ,5,7,7-hexachlorofluorescein succinimidyl ester) PET tag labeled to the 5' end of the reverse primer. In a 20uL total PCR reaction volume, 8uL purified sample DNA was added to 12uL reaction mix (10uL 2x AB Environmental Master Mix (Applied Biosystems, Foster City, CA), 0.5uL forward primer, 0.5uL reverse primer, 1uL dH_2O). Samples were run on StrataGene Mx3005P machine (Agilent Technologies, Santa Clara, CA) with conditions of: 10min at 95^{o}C followed by 45 cycles of 10s at 95^o, 40s at 60^o. Data were analyzed with MxPro software.

Protein Elution from Dried Blood Spots. For each individual, a 6mm diameter blood spot (equivalent to 14uL whole blood) was placed into 140uL elution buffer (PBS, 0.05% Tween-20, 0.05% sodium azide) in a 96-well plate. Plates were shaken at 60 rpm at room temperature overnight. Eluate was stored at 4^{o}C until further use.

Development of Positive and Negative Anti-Malarial IgG Controls. Initial standard curves for ELISA assays for all 4 *P. falciparum* antigens were developed using positive controls of serum samples of persons from geographical regions endemic or hyperendemic to *P. falciparum* malaria. Highly-reactive serum samples from Kenya, Bolivia, Guyana, Brazil, India were pooled in order to create a "hyperimmune serum" pool which was then diluted in whole blood (from the Memphis, TN bloodbank) and spotted onto Whatman 903 booklets for later extraction and ELISA

assays. Negative controls were produced from blood from Memphis bloodbank which had shown no presence of antibodies to malarial antigens.

Enzyme-Linked Immunosorbant Assay (ELISA) to Quantify Anti-Malarial IgG Antibodies. The malarial antigens MSP-$1_{42(D)}$, MSP-$1_{42(F)}$, MSP-1_{19}, and AMA-1 were all obtained as a generous gift from Dr. Chris Drakely, London School of Hygiene and Tropical Medicine, London, UK. A novel protocol was developed for ELISA quantification of anti-malarial IgG antibodies and can be found in Appendix C. A sample positive for IgG antibodies against one of the malarial antigens showed an optical density (OD) reading above three times the average of the nonimmune Memphis samples. For a rendering of positivity for an individual, an individual's sample must show OD "positivity" for any 2 of the MSP-1 fragments (MSP-$1_{42(D)}$, MSP-$1_{42(F)}$, MSP-1_{19}), or for AMA-1 alone, or both of these criteria in conjunction. The elucidation of this seropositivity criteria is given in this manuscript's Results and Discussion sections.

RESULTS

Figure 7 shows the location of study sites for the PSI-conducted 2012 Haiti TRaC survey. Sites were intentionally chosen to represent a diverse sampling of the nation and cover the entire area of the country. In areas of higher population density, more sites were chosen in order to attempt to maintain a consistent percentage of the total population which was sampled over Haiti's entire geographical region. This strategy is easily displayed by comparing the departments of Ouest and Nord-Est. The densely-populated Ouest department contains 7 of the top 10 largest Haitian cities, and accounts for around 30% of Haiti's total population (34). For the 2012 TRaC survey, the Ouest department also contained 22 of the 62 SDE locations (35% of study sites). In contrast, the Nord-Est department contains only 3% of Haiti's population (34), and 2 SDE sites for the 2012 TRaC survey (3% of study sites). Even within a department, the number and density of sites were allocated according to the department's population density. For example, in the Nord department, 2 of the 5 SDE sites are within Cap-Haïtien, which is the largest city within the department. Using these aforementioned strategies, data gathered from the 2012 TRaC survey can more confidently be translated to estimates of parasite rate and malaria seropositivity not only with an individual department, but for the country as a whole. Table 1 shows the number of SDE study sites within each department, the percentage of the Haitian population within that department, and the proportion of SDE sites for that department compared to total number of SDE sites (which is 62).

As asymptomatic persons with malaria infection are still able to transmit the parasite to mosquitoes during a blood meal, it is important to be able to identify these asymptomatic cases in order to reduce the possibility of continued malaria cyclic transmission between humans and the mosquito vector. For diagnostic purposes, only cases showing clinical symptoms would be of relevance, but surveillance studies require a method of high sensitivity in order to accurately identify possible foci of intense parasite prevalence. The purpose of choosing the sensitive

method of PCR for this survey was to be able to detect very low parasite densities which would likely not cause symptomatic infection, but still transmit gametocytes to mosquitoes. Previous studies with the PCR primers used for the 2012 Haiti TRaC Survey had shown a sensitivity and specificity of both 100%, with a limit of detection around 3.2 parasites/uL of blood (16, 35). Before starting analysis on the Haiti samples, it needed to be confirmed that a similar (or better) limit of detection could be met when samples were run on our machinery. As shown by Figure 8, PCR reactions gave very clear products when standards of known parasite density were run from the concentrations of 10,000 to 4.88 parasites per microliter (p/uL) of sample. As the samples were run in duplicate, clear bi-partite PCR products were shown for all replicates to the concentration of 4.88p/uL. However, when parasite density reached lower than 4.88p/uL, it was found that primers began to lose their sensitivity, and that one replicate could be positive while the other replicate negative. Using these data, we are able to confidently say that the primers used in this study were able to reliably detect between 4.88 and 2.44 malaria parasites per microliter of blood.

Though our analysis of the parasite limit of detection for these primers was found to be very low, only one sample was found to be positive by PCR for the 2012 TRaC survey. In total, 581 samples were analyzed, which would give a crude parasite rate (PR) of 0.17%. As a previous 2011 TRaC survey (manuscript in preparation) had also shown a low PR for Haiti, this estimate of low PR for the nation is not surprising. The sample found to be positive for malaria parasites was from the Ouest department at the location of SDE 59. This corresponds to the city of Petit Goave which is on the coast of the Gulf of Gonâve. The geographical location for the origin of this positive sample is shown on the Haitian map in Figure 9.

As PCR analysis for active infection can give an estimate for what percentage of the population is infected at any one given time throughout the year, the analysis of antibodies against malarial antigens gave a much broader picture to the history of infection for humans in a geographical area. Seropositivity curves were made by presence of antibodies to the *P.*

falciparum merozoite surface protein 1 (MSP-1) or apical membrane antigen 1 (AMA-1). Since three antigens were used to represent an antibody response to MSP-1 (the fragments MSP-$1_{42(D)}$, MSP-$1_{42(F)}$, and MSP-1_{19}) the presence of antibodies to at least two of these three fragments caused an individual sample to be seropositive. In contrast, positive antibody response to the AMA-1 protein, itself, was the only criteria to classify an individual sample as 'AMA-1 seropositive'. Generally, estimates of the seroconversion rate (λ) are given as the risk of moving from seronegativity to seropositivity within the course of a year's time. However, due to the paucity of data for each age, serology data from the ELISA assays had to be treated in a categorical manner with six different age categories of: 0-5, 6-10, 11-20, 21-30, 31-40, >40 years of age. Instead of generating estimates of seroconversion rates per annum, rates were estimated to predict the acquisition of seropositivity as you transitioned from one category to the next. The rate will vary between -1 and 1 with positive values showing an increase in the likelihood of seroconversion as one ages, and negative values showing a decreased likelihood over time. A rate of 1 would indicate certainty of seroconversion from one age category to another, and a rate of 0 would indicate no change between categories. Since seroreversion (the loss of antibodies over time) is possible, seroconversion rates below 0 would indicate the near complete absence of malaria, and loss of antibody response by the population (38).

Figure 10 illustrates the aggregate seroprevalence curves for the nation of Haiti based on the 491 samples analyzed. As discussed earlier, curves are separated into seropositivity to MSP-1 antigens or the AMA-1 antigen since both proteins are known to elicit unique humoral responses. As would be expected, the aggregate curves in Figure 10 show an increasing seroprevalence for both curves as age categories increase. The two seropositivity curves showed a relatively close estimate for the seroconversion rate with the curve for MSP-1 antigens showing a rate of 0.147 and the curve for AMA-1 showing a rate of 0.083. With both curves showing a rate greater than zero, the data are indicative of increased likelihood of becoming seropositive as the Haitian individual ages. Surprisingly, both curves showed very high estimates of y-intercepts, with the

MSP-1 curve giving an intercept of 0.429 and the AMA-1 curve giving an intercept of 0.275. Both of these estimates are quite high considering the suspected low endemicity of *P. falciparum* malaria in Haiti and may be indicative of high susceptibility of infants to mosquito bites. Both MSP-1 and AMA-1 curves showed different levels of predictive power with the MSP-1 curve giving an R^2 value of 0.767 and the AMA-1 curve with a lower R^2 value of 0.488.

Seroprevalence curves were also separated by the ten Haitian departments. Figure 11 shows the department-specific results, which is also summarized in Table 2. Most seroprevalence curves showed a very normal pattern of increasing estimates of seroconversion as age categories increased, but curves occasionally gave negative seroconversion estimates. The most extreme estimates for seroconversion rates for MSP-1 protein were given by the Grand'Anse department with an estimate of 0.499 and the Sud department with an estimate of -0.296. Also, widely varying were the estimates for the y-intercept with the Nord-Est department showing an estimate of 0.977 and the Sud-Est department with an estimate of -0.035. These large differences in the estimates in seroconversion rate and y-intercepts among departments in Haiti point towards a heterogenous distribution of *P. falciparum* malaria throughout the country.

DISCUSSION AND FUTURE DIRECTIONS

Since the advent of more coordinated malaria control efforts in the past three decades, no reliable malaria map has been generated in which to focus efforts for eventual elimination from Haiti. Every year, the World Health Organization compiles a report on all malaria endemic countries showing estimated *P. falciparum* endemicity within each country, and statistics on case counts and nationwide mortality (2). This report also includes a map of the country with the distribution of confirmed malaria cases. Due to low reporting and minimal reporting infrastructure, the WHO-generated map provides no informative data in order to address malaria control. Other groups have used these vague estimates to assess the possible clinical burden of malaria upon the country (15), and, as shown by Figure 5, a very low amount of resolution is currently available. As Haiti's current status is in the malaria "Control" phase without a great amount of oversight, the impact of the country's efforts has "insufficiently consistent data to assess trends"(2). In simple terms, the amount of data that has been generated regarding the status and severity of *P. falciparum* malaria in Haiti is vanishingly small, and cannot be reliably used by public health officials to target areas of the nation needed focused malaria control efforts. This manuscript reports on samples gathered from multiple locations within each of the 10 Haitian departments in order to add additional information to the attempt to gain a reliable parasite rate and seroconversion rate for the nation of Haiti.

In trying to estimate the national malaria burden, the measurement of *P. falciparum* parasite rate (PR) has advantages and disadvantages. One of the major advantages PR affords for the purposes of surveillance is the ability to get a clear and accurate point estimate for the study population (39). In this study, PR was generated by the evaluation of *P. falciparum* DNA from dried blood spots (DBS). Recently, biomedical companies have made great improvements in the filter paper used to collect DBS, and DNA and proteins can now remain quite stable on filter paper for many months at room temperature, and for even longer periods of time when refrigerated (40). When coupled with a highly-sensitive detection method such as PCR, one can

have a high degree of certainty that if a person was infected with malarial parasites, they would be found positive by laboratory methods. Before analysis of field samples, it was important to verify that the PCR methodology used for the 2012 TRaC survey would be highly sensitive, and be able to detect blood parasites that would be missed by the conventional detection method of microscopy. This was indeed the case, as we found that the PCR protocol to be used for DBS was able to reliably detect somewhere between 4.88 and 2.44 parasites/uL whole blood. This finding was also consistent with the inaugural publishing of this PCR protocol showing a limit of detection around 3.2 parasites/uL (16). For the 2012 TRaC Survey, the PR for Haiti was found to be very low at 0.17%. This low PR was not unexpected, as a previous survey conducted by the CDC in 2011 found a PR of around 0.4% (data unpublished). In this study, only one sample was found to be positive for *P. falciparum* DNA although 581 samples were analyzed. Many factors could possibly explain why only one sample was found to be positive. Though consistent and reliable reporting data has not historically existed for Haiti, it is generally assumed that this nation is a low to very low endemic setting for malaria parasites. Once *P. falciparum* endemicity reaches a very low level, those who are actually infected with the parasite likely have very low levels of parasites in their bodies due to the biological dynamics of *falciparum* infection (Figure 4). The purpose of calculating the true PR for an area is to obtain knowledge of an infection, no matter how low the parasite levels. Our PR of 0.17% could be an underestimate due to possible degradation of parasite DNA between the time of sample collection and sample processing, especially when the samples were at warmer temperatures in the field. Another possibility is that our PCR methodology is simply not sensitive enough, and some samples processed had parasitemia lower than 2.44 parasites/uL. A parasitemia of this level (or lower) would not be detected by the PCR assay, and would be counted as negative, even though that individual harbored an infection with *P. falciparum*. Since a 100% sensitive assay would (hypothetically) be able to detect 1 parasite in an entire person, the PCR assay used for the 2012 TRaC Survey has an unknown sensitivity which is likely high, but certainly under 100%. Though the sensitivity for

this assay is under 100%, it can be stated with a high degree of certainty that the PCR methodology used for the Survey was 100% specific. With only one confirmed positive sample, that sample was assayed with PCR analysis multiple times, and a replicate blood spot for that individual was subjected to a repeat of DNA extraction and PCR analysis to verify true positivity.

Another advantage to calculation of the PR is the easy explanation to what that percentage actually means. With our calculation of a PR of 0.17%, the simple interpretation is that 0.17% of the participants in the 2012 TRaC Survey were found to have active *P. falciparum* infection. One of the difficult aspects, however, is the translation of this percentage to the entire nation of Haiti. Obviously, with only one positive sample in the Study, it would be imprudent and impossible to translate the results to the 10 different departments in Haiti. Nine of the departments would have a 0% PR, which is certainly not true. The study design for this survey intentionally chose multiple localities within each of the departments, and also selected for urban and rural areas to attempt to get a more representative sampling of the population. The cross-sectional nature of the study is very appropriate considering the malaria dynamics within Haiti. The peak season of malaria transmissibility in Haiti occurs during the major rainy season from October to December (31). The 2012 Haiti TRaC Survey took place during this season of peak transmission, but the vast majority of samples were collected in late November and December. Since *P. falciparum* infection will last for multiple weeks if left untreated (23), someone infected in October or November will most likely still harbor the parasite during the apex of sample collection and have ample parasite DNA in their blood. With the application of all of this information, calculating a PR for Haiti can give public health officials a confident estimate of the intensity of infection for the year 2012, and public health officials can be optimistic that the estimate generated from this study is so low. Future studies should gather more samples from different areas within departments in order to be able to more reliably translate the findings to the country as a whole.

Since historically reliable reporting data on *P. falciparum* parasite rate in Haiti does not exist, one strategy for overcoming this adversity is to look at biological factors predicting malaria infection that are long-lasting. For the 2012 Haiti TRaC Survey, this was accomplished by looking at IgG antibodies against *P. falciparum* which are known to be present in the blood of a previously-infected person for years, if not decades (13,14). These antibodies are highly-stable on DBS, and the highly-sensitive laboratory assay of ELISA shows an extremely low limit of detection. The four *P. falciparum* antigens used in this study are some of the most thoroughly studied in the field of malaria research, and MSP-1 antigens were initially considered as vaccine candidates for a malaria vaccine (41). The antigens MSP-$1_{42(D)}$, MSP-$1_{42(F)}$, and MSP-1_{19} are all fragments of the MSP-1 protein and were used to collectively estimate an immune response to *P. falciparum* MSP-1. All of the MSP-1 fragments as well as AMA-1 are well-known to generate reliable and robust immune responses in the human host, which is why they were included in this study.

Though a seropositivity percentage can be calculated for a population (number seropositive divided by sample size x 100%), this number gives limited information due to the nature of the antibody response. A much more useful analysis of serological data is to plot seropositivity versus age and generate a best-fitting seropositive curve. The logarithmic equation for this curve is known as the seroconversion rate, and is abbreviated by the Greek letter lambda. Since antibody titers remain detectable for so long after *P. falciparum* infection, seropositivity curves, themselves, have two aspects which can be informative: the initial rise in percent seropositive from 0-10 years of age and the plateau which estimates regional endemicity. Children are born seronegative, so assessing how quickly children become seropositive in their first 10 years of life helps to estimate malaria's force of infection (20). The plateau of a seropositivity curve tends to approach a straight line when you get to older ages, and this can help estimate how much of the population will be infected with malaria in their lifetime. For the 2012 Haiti TRaC survey, two seropositivity curves were generated for antibody responses against the

P. falciparum MSP-1 and AMA-1 proteins. The rationale for partitioning seropositivity curves into different curves for MSP-1 and AMA-1 comes from research behind the human immune response to these two *P. falciparum* antigens. Multiple groups have shown that the human humoral response to these antigens is different, not in terms of magnitude of response, but in the temporal nature. Antibodies against MSP-1 and its fragments tend to develop more slowly, but increase with repeated exposure and have a longer duration (36, 37). In contrast, the humoral response against AMA-1 is more robust early in the infection, and quickly wanes over time. One study found a greater than four-fold decrease in the presence of IgG antibodies against AMA-1 a short 5 months after the initial high antibody titer (36).

The 2012 Haiti TRaC Survey found a high percentage of the Haitian population that was seropositive for antibodies to both the MSP-1 and AMA-1 proteins. As expected, the seroprevalence for AMA-1 was lower at each age category, adding support to previous groups' findings that the AMA-1 antibody response is more short-lived. The seroconversion rate was nearly twice as high for MSP-1 (0.147) than for AMA-1 (0.083). Highly unexpected was the high estimates for the y-intercept for both seropositivity curves with MSP-1 providing an estimate of 0.429 and AMA-1 giving an estimate of 0.275. Again, an infant is born seronegative, and can only become seropositive by infection with *P. falciparum.* These high estimates for the y-intercepts indicate a very high rate of seroconversion during the first few years in life. A high rate of seroconversion among the young persons in the population is indicative of a high force of infection, high malaria endemicity, or both. Since nearly 500 samples were used for the 2012 Haiti TRaC Survey from different regions throughout the nation, it is not unreasonable to assume that malaria endemicity is possibly higher than previously thought. Before conclusions can be drawn, some caveats to this analysis should be mentioned. One of the major assumptions of serological data is that malaria endemicity is consistent among transmission seasons, and that the force of infection would also remain relatively constant (39). If, prior to the 2012 Survey, unusually high transmission seasons had occurred for the previous 4 or 5 years, the entire

population would have a more similar seroprevalence, regardless of age. This would also force the seroprevalence curve to be composed much more of the plateau phase and much less of the initial rise phase for the younger age groups. Interestingly, this is exactly what we see in our seroprevalence curves, with young ages showing high seroprevalence. Serology data was also separated among the 10 Haitian departments. Likely due to the paucity of data for each age group within each department, seroprevalence curves showed little predictive power with low R^2 values for the majority of the departments. The one exception was the Centre department, which gave an excellent R^2 value for MSP-1 (0.801) and good value for AMA-1 (0.595). Interestingly, both of these curves for the Centre department also gave low estimates for the y-intercept which may point to a more consistent *P. falciparum* endemicity over time.

Though currently considered in a malaria control phase, the island nation of Haiti is a prime candidate to move towards malaria pre-elimination. One of the more striking advantages afforded to Haiti is its geographical location of being completely surrounded by water with the exception of its shared eastern border with the Dominican Republic. In this isolated state, the possibility of reintroduction is greatly reduced due to these geographical boundaries (30). Two other advantages Haiti currently has in working towards elimination are its near homogeneity with respect to the *P. falciparum* malaria species and no evidence supporting introduction or development of chloroquine resistance in Haiti (33). Future surveillance studies should continue to attempt to sample from all Haitian departments and include urban as well as rural areas for sample collection. One of the major limitations of the 2012 Haiti TRaC survey was the limited amount of samples that were collected and able to be analyzed for the parameters of our interest. Greater amount of sample collection in the future will allow for more robust serological analysis by allowing adequate population of the age groups when generating seroprevalence curves. In addition, greater sample size can lead to more confidence in estimating the true parasite rate each year.

REFERENCES

1) Wellems, TE, Hayton, K, Fairhurst, RM. The impact of malaria parasitism: from corpuscles to communities. *J Clin Invest* 2009; 119(9):2496-2505.

2) World Health Organization: WHO Global Malaria Programme. World Malaria Report 2013. Published at the National Press Club, Washington DC on Dec 11, 2013.

3) Hart, DL. The origin of malaria: mixed messages from genetic diversity. *Nat Rev Microbiol*. 2004; 2(1):15-22.

4) Guerra, F. The European-American exchange. *Hist Philos Life Sci*. 1993; 15(3):313-327).

5) Carter, R, Mendis, KN. Evolutionary and historical aspects of the burden of malaria. *Clin Microbiol Rev* 2002; 15(4):564-594.

6) Gething, PW, Patil, AP, *et al*. A new world malaria map: *Plasmodium falciparum* endemicity in 2010. *Malaria J*. 2011;

7) Mejia, R, Booth, GS, *et al*. Peripheral blood stem cell transplant-related Plasmodium falciparum infection in a patient with sickle cell disease. *Transfusion*. 2012; 52(12):2677-2682.

8) Noubouossie, D, Tagny, CT, *et al*. Asymptomatic carriage of malaria parasites in blood donors in Yaounde. *Transfus Med* 2012; 22(1):63-67.

9) Lombardo, F, Lanfrancotti, A, *et al*. At the interface between parasite and host: the salivary glands of the African malaria vector Anopheles gambiae. *Parassitlolgia*. 2006; 48(4):573-580.

10) Kappe, SH, Vaughan, AM, *et al*. That was then but this is now: malaria research in the time of an eradication agenda. *Science*. 2010; 328(5980):862-866.

11) Kwiatkowski, DP. How malaria has affected the human genome and what human genetics can teach us about malaria. *Am J Human Gen*. 2005; 77(2):171-192.

12) Gupta, S., Snow, RW, *et al*. Immunity to non-cerebral severe malaria is acquired after one or two infections. *Nature Med*. 1999; 5(3):340-343.

13) Langhorne, J, Ndungu, FM, Sponaas, A, Marsh, K. Immunity to malaria: more questions than answers. *Nature Immunol*. 2008; 9(7):725-732.

14) Ndungu, FM, Lundbloom, K, *et al*. Long-lived *Plasmodium falciparum* specific memory B cells in naturally exposed Swedish travelers. *Eur J Immunol*. 2013; 43(11):2919-2929.

15) Hay, SI, Okiro, EA, *et al*. Estimating the global clinical burden of *Plasmodium falciparum* malaria in 2007. *PLOS Med*. 2010; 7(6):e100029.

16) Lucchi, NW, Narayanan, J, *et al*. Molecular diagnosis of malaria by photo-induced electron transfer fluorogenic primers: PET-PCR. *PLOS One*. 2013; 8(2):e56677.

17) Baidjoe, A, Stone, W, *et al*. Combined DNA extraction and antibody elution from filter papers for the assessment of malaria transmission intensity in epidemiological studies. *Malar J*. 2013; 12:272.

18) Cook, J, Reid, H, *et al*. Using serological measures to monitor changes in malaria transmission in Vanuatu. *Malar J*. 2010; 9:169.

19) Cook, J, Kleinschmidt, I, *et al*. Serological markers suggest heterogeneity of effectiveness of malaria control interventions on Bioko Island, equatorial Guinea. *PLOS One*. 2011; 6(9):25137.

20) Corran, P, Coleman, P, Riley, E, Drakely, C. Serology: a robust indicator of malaria transmission intensity? *Trends Parasitol*. 2007; 23(12):575-582.

21) The silent threat: asymptomatic parasitemia and malaria transmission. *Expert Rev Anti Infect Ther*. 2013; 11(6):623-639.

22) Batista-dos-Santos, S, Raiol, M, *et al*. Real-time PCR diagnosis of *Plasmodium vivax* among blood donors. *Malaria J*. 2012; 11:345.

23) Okell, LC, Bousema, T, *et al*. Factors determining the occurrence of submicroscopic malaria infections and their relevance for control. *Nat Commun*. 2012; 3:127.

24) Cheng, Z, Sun, X, *et al*. A novel, sensitive assay for high-throughput molecular detection of plasmodia for active screening of malaria for elimination. *J Clin Microbiol*. 2013; 51(1):125-130.

25) Kamau, E, Tolbert, LS. Development of a highly sensitive genus-specific quantitative reverse transcriptase real-time PCR assay for detection and quantitation of plasmodium by amplifying RNA and DNA of the 18S rRNA genes. *J Clin Microbiol*. 2011; 49(8):2946-2953.

26) Kilama, WL. Health research ethics in public health: trials and implementation of malaria mosquito control strategies. *Acta Trop*. 2009; 1:S37-47.

27) Feachman, RG, Phillips, A, *et al*. Shrinking the malaria map: progress and prospects. *Lancet*. 2010; 376:1566-1578.

28) Luby, JP, Collins, WE, Kaiser, RL. Persistence of malarial antibody. Findings in patients infected during the outbreak of malaria in Lake Vera, California, 1952-1953. *Am J Trop Med Hyg*. 1967; 16(3):255-257.

29) Zucker, JR. Changing patterns of autochthonous malaria transmission in the United States: a review of recent outbreaks. *Emerg Infect Dis*. 1996; 2(1):37-43.

30) Kaneko, A, Taleo, G, *et al*. Malaria eradication on islands. *Lancet*. 2000; 356:1560-1564.

31) Roberts, L. Elimination meets reality in Hispaniola. *Science*. 2010; 328:850-851.

32) Malaria Surveillance – United States. Data collected from years 2001 to 2011. *MMWR*.

33) Keating, J, Krogstad, DJ, Eisele, TP. Malaria elimination on Hispaniola. *Lancet Infect*. 2010; 10:291-293.

34) GeoNames Online database. Haiti-Largest Cities. Accessed at: <http://www.geonames.org/HT/largest-cities-in-haiti.html>

35) Talundzic, E, Maganga, M, *et al.* Field evaluation of the photo-induced electron transfer fluorogenic primers (PET) real-time PCR for the detection of Plasmodium falciparum in Tanzania. *Malar J*. 2014; 13(31):13-31.

36) Nhabomba, AJ, Guinovart, C, *et al.* Impact of age of first exposure to *Plasmodium falciparum* on antibody responses to malaria in children: a randomized, controlled trial in Mozambique. *Malaria J*. 2014; 13(121)

37) Moncunill, G, Mayor, A, *et al*. High antibody responses against Plasmodium falciparum in immigrants after extended periods of interrupted exposure to malaria. *PLoS One* 2013; 8(8):e73624.

38) Kusi, KA, Bosomprah, S, *et al*. Anti-sporozoite antibodies as alternative markers for malaria transmission intensity estimation. *Malaria J.* 2014; 13(1).

39) Tusting, LS, Bousema, T, *et al*. Chapter Three – Measuring changes in *Plasmodium falciparum* transmission: precision, accuracy, and cost of metrics. *Advances in Parasitol*. 2014; 84:151-208.

40) Corran, PH, Cook, J, *et al*. Dried blood spots as a source of anti-malarial antibodies for epidemiological studies. *Malaria J.* 2008; 7(195).

41) Holder, AA, Guevara JA, *et al*. Merozoite surface protein 1, immune evasion, and vaccines against asexual blood stage malaria. *Parassitologia* 1999; 41(1-3):409-414.

FIGURES AND FIGURE LEGENDS

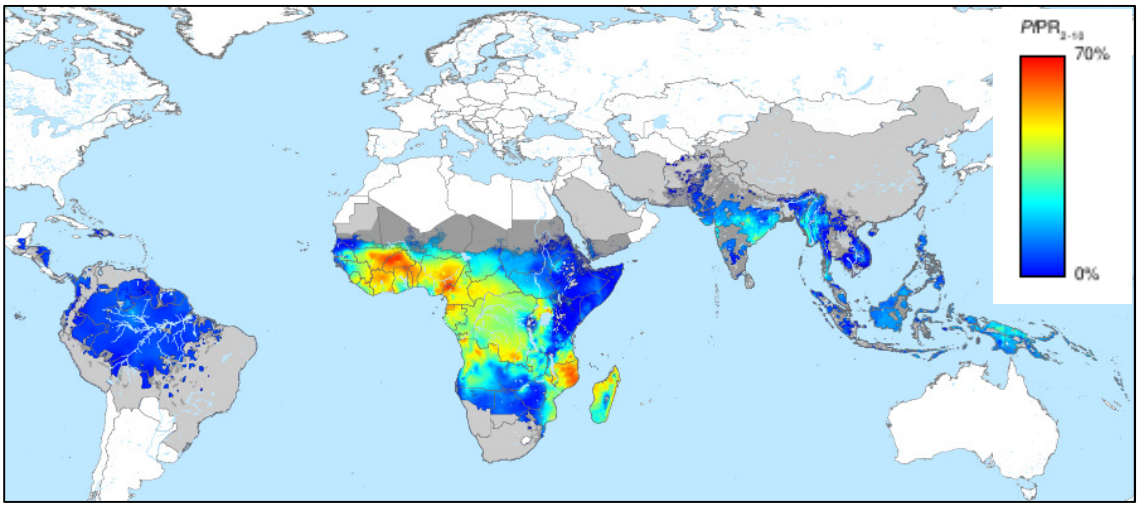


Figure 1. Global *Plasmodium falciparum* endemicity in 2010. Heat map showing mean estimates of parasite rate (*Pf*PR) for 2010 by model-based geostatistical prediction. Parasite rate gives a percentage of the population which harbors the parasite at any one time. Dark grey areas represent stable transmission with *Pf*PR greater than 0.1 per annum. Map from Ref. 6.

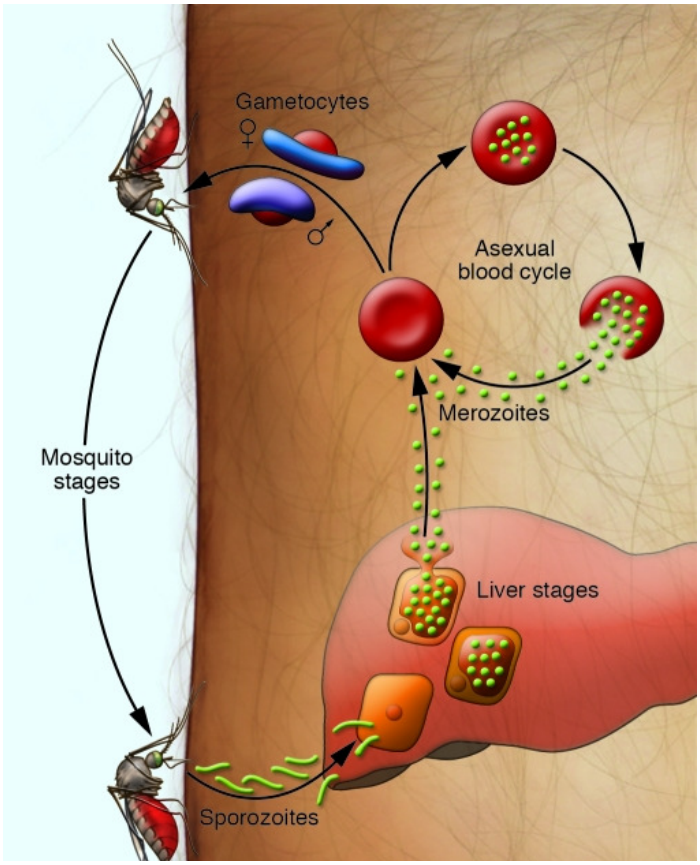


Figure 2. Lifecycle of *Plasmodium falciparum* within the human host. The simplified schematic shows initial infection with malarial sporozoites, migration to the liver, and release of merozoites into the systemic circulation to begin blood stage infection. During the cyclical blood stage infection, some parasites replicate as male or female gametocytes where they are then taken up by a biting mosquito where they will undergo sexual reproduction. Schematic from Ref. 1.

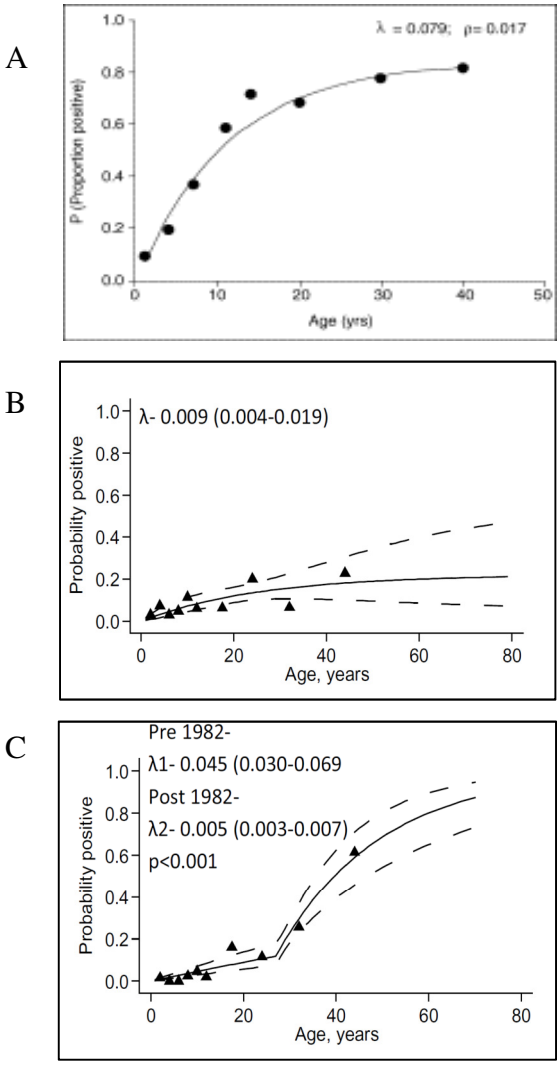


Figure 3. Representative seropositivity plots showing proportion of a study population positive for anti-malarial antibodies. A) Example of high-transmission setting in central Africa (Ref. 20). B) Low transmission setting on one of the islands of Vanuatu (Ref. 18). C) Area of initial high transmission on Vanuatuan island that was stymied by successful malaria control effort (Ref. 18). Each plot also provides the estimate for the seropositivity rate for an individual per year as indicated by the letter lambda. Notice how plot 'C' provides a pre- and post-intervention seropositivity rate.

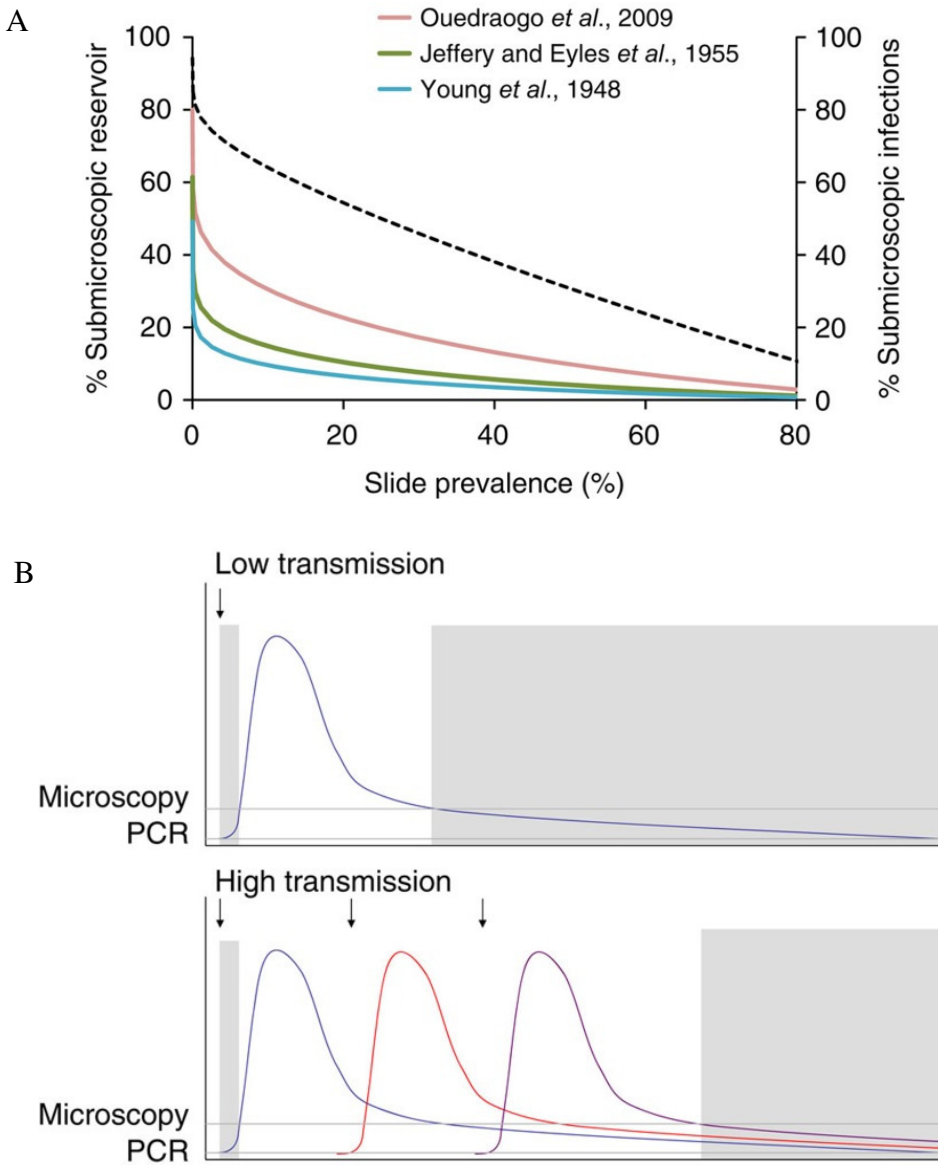


Figure 4. The importance of detecting sub-microscopic malarial infections. A) Three independent studies showing the lowered submicroscopic reservoir as microscope slide prevalence is increased. Increased slide prevalence is also an indicator of high malaria transmission intensity. B) Typical *Plasmodium falciparum* parasite load during the course of infection. In low transmission settings, the majority of the time a person is infected will be undetectable by microscopy, as indicated by the gray zones. High transmission settings allow more possibilities for case detection. Plots from Ref. 23.

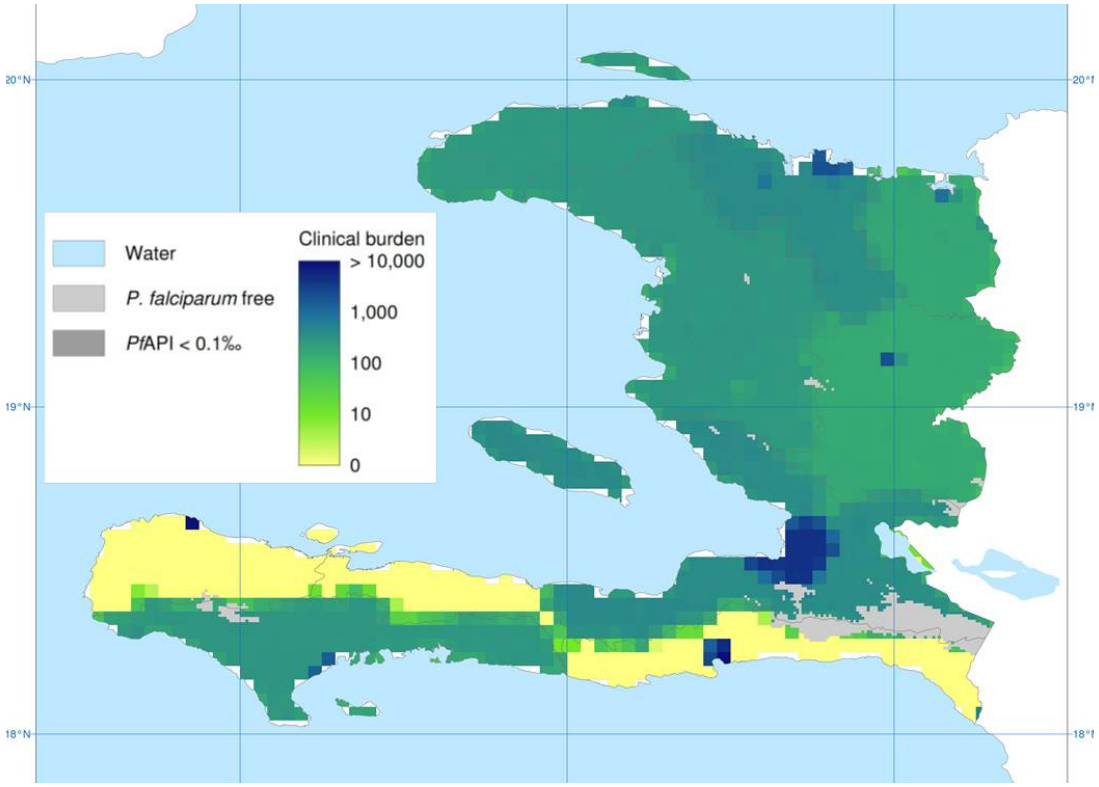


Figure 5. Estimates of clinical burden in human cases of *P. falciparum* in the nation of Haiti by location. Predictions are based on a Bayesian geostatistical model that uses predictions of malaria endemicity, relationship between prevalence and clinical incidence, and population estimates. *Pf*API: *Plasmodium falciparum* annual percentage incidence. Schematic from Ref. 15.

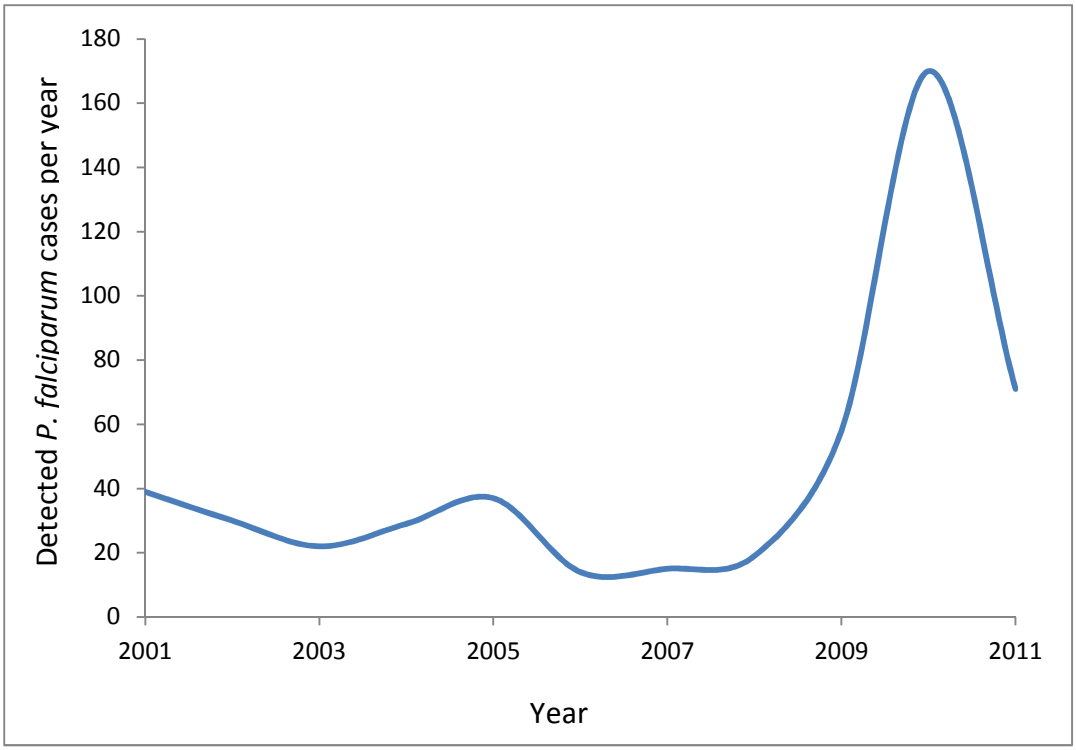


Figure 6. Number of imported *P. falciparum* cases from Haiti to the United States from 2001 to 2011. Data from *MMWR*: Malaria surveillance – United States.

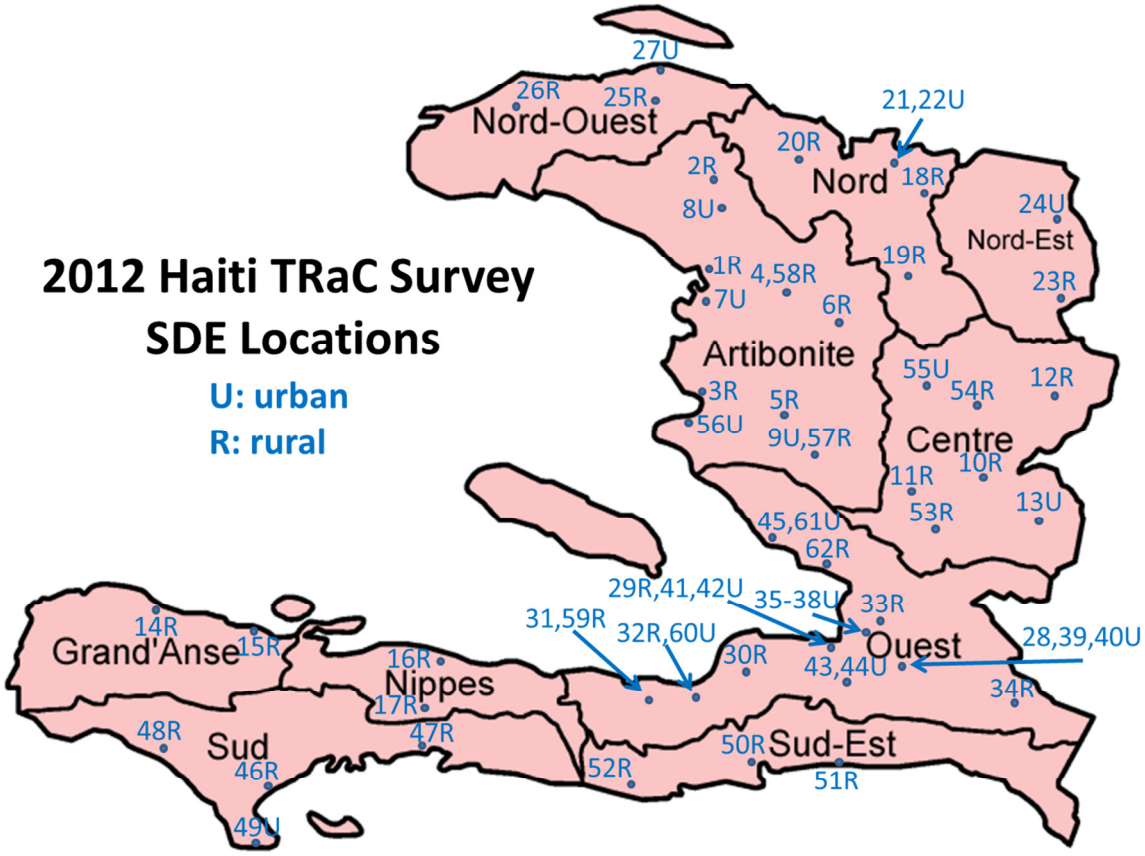


Figure 7. Locations of study sites for the 2012 Haiti Malaria TRaC Survey as designated by PSI. Haitian department names indicated in black text and study sites in light blue with designation of a rural or urban site. SDE: sélectionner par department.

Table 1: Location of SDE Sites within Each Department and Proportion of Haitian Population Represented

Haitian Department	Population* (% total)	SDE sites (% total)
Nord-Ouest	445,080(5.3)	3(4.8)
Nord	872,200(9.3)	5(8.1)
Nord-Est	300,493(3.9)	2(3.2)
Artibonite	1,070,397(11.2)	12(19.4)
Centre	565,043(6.4)	7(11.3)
Ouest	3,093,698(30.5)	22(35.5)
Gran'Anse	337,516(4.2)	2(3.2)
Nippes	263,000(3.5)	2(3.2)
Sud	745,000(8.1)	4(6.4)
Sud-Est	518,200(5.9)	3(4.8)
	8,210,627 (100)	**62 (100)**

* Population data obtained from Ref. 34.

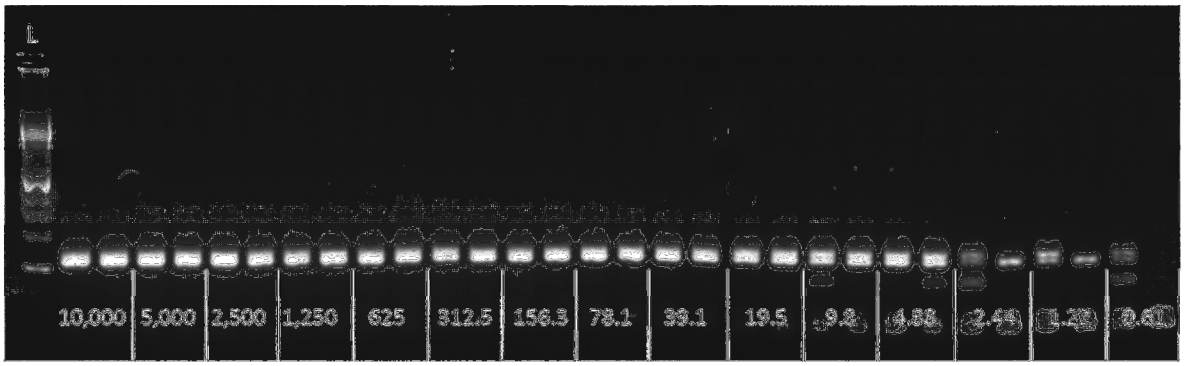


Figure 8. Validation of limit of detection for primers used for parasite detection in the 2012 Haiti Malaria TRaC Survey. PCR reactions were run in duplicate on samples of known parasite densities in two-fold serial dilutions from 10,000 to 0.61 parasites/uL sample. Shown is the agarose gel electrophoresis with the PCR product from the reaction. L: 100 base-pair DNA ladder to verify bi-partite band between 100 and 150 base pairs.

Figure 9. Participant sample found positive for malaria DNA by PCR. Of 581 DBS samples assayed for the 2012 TRaC survey, only one sample was found to be positive for malaria DNA in the Ouest department in the city of Petit Goave.

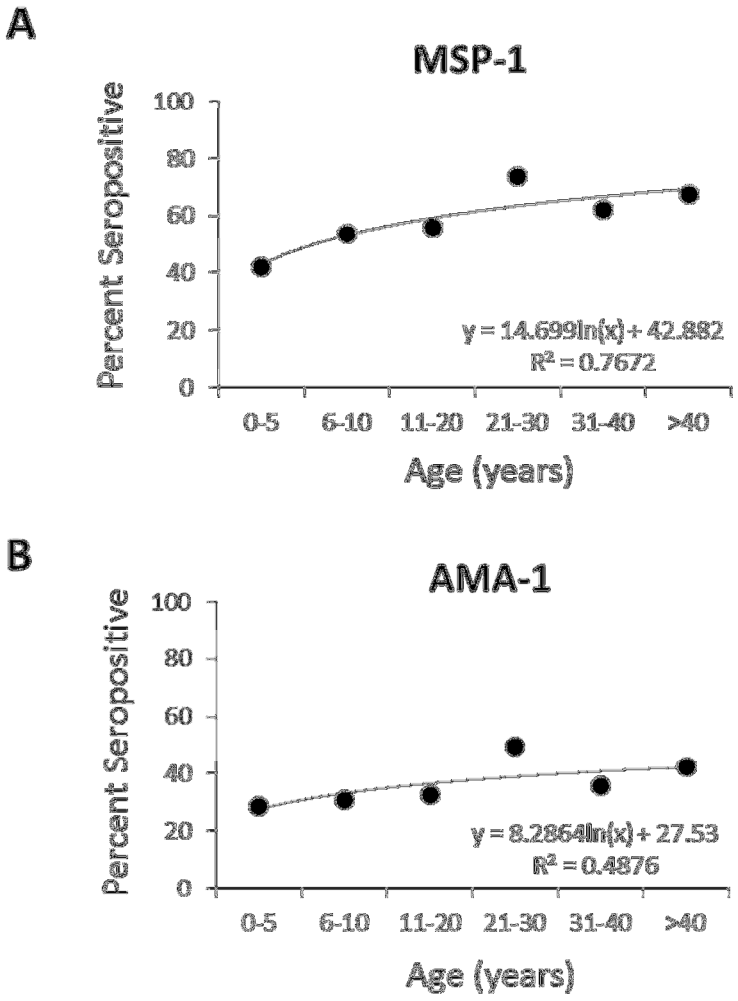


Figure 10. Seroprevalence curves for the nation of Haiti based on percent population with anti-malarial antibodies. A) Curve generated by positivity to at least 2 of the 3 MSP-1 antigens used in the ELISA assay. B) A more conservative curve generated by the populations' positivity to the AMA-1 antigen.

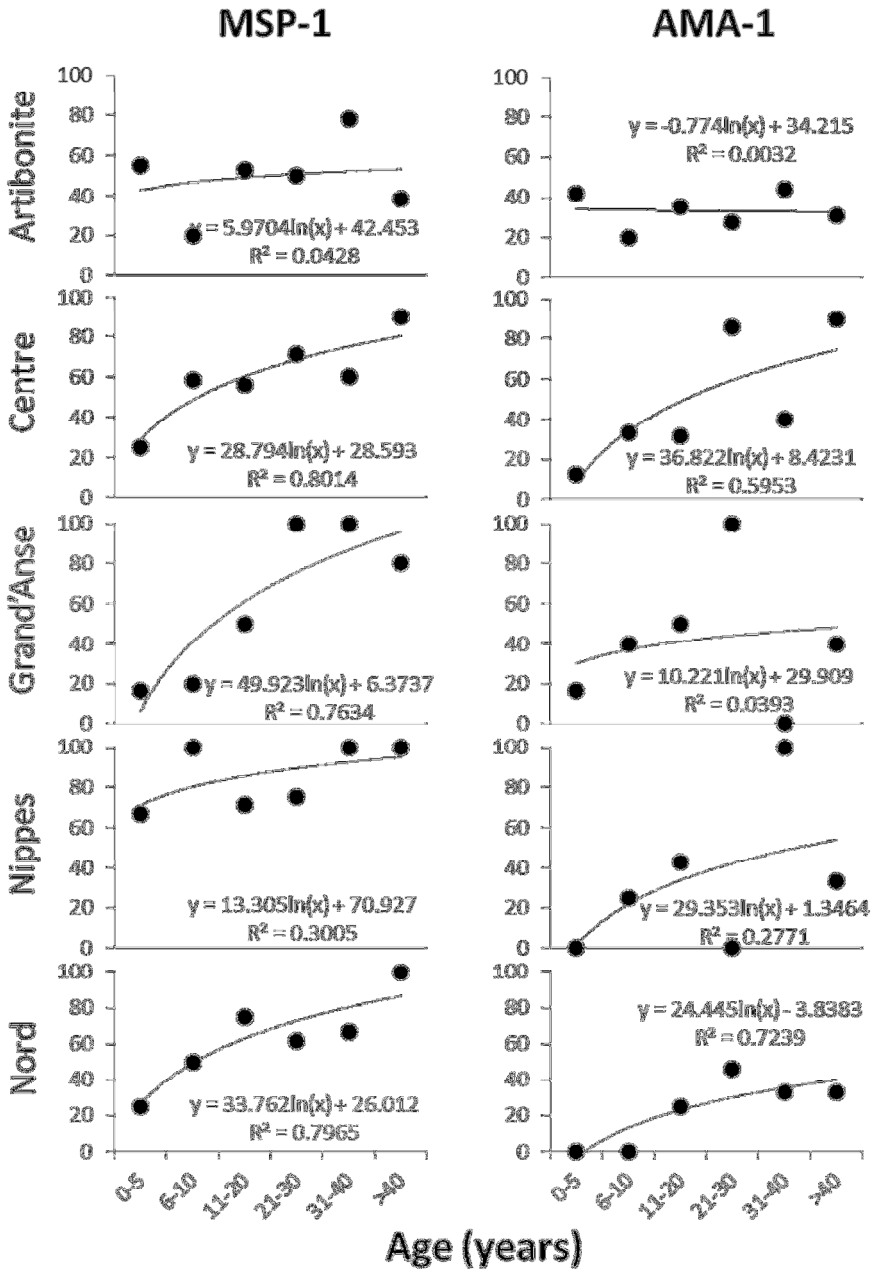


Figure 11. Seroprevalence curves for Haitian departments based on percent population with anti-malarial antibodies. The y-axis displays percentage of the department's seropositive population whereas the x-axis displays age calipers.

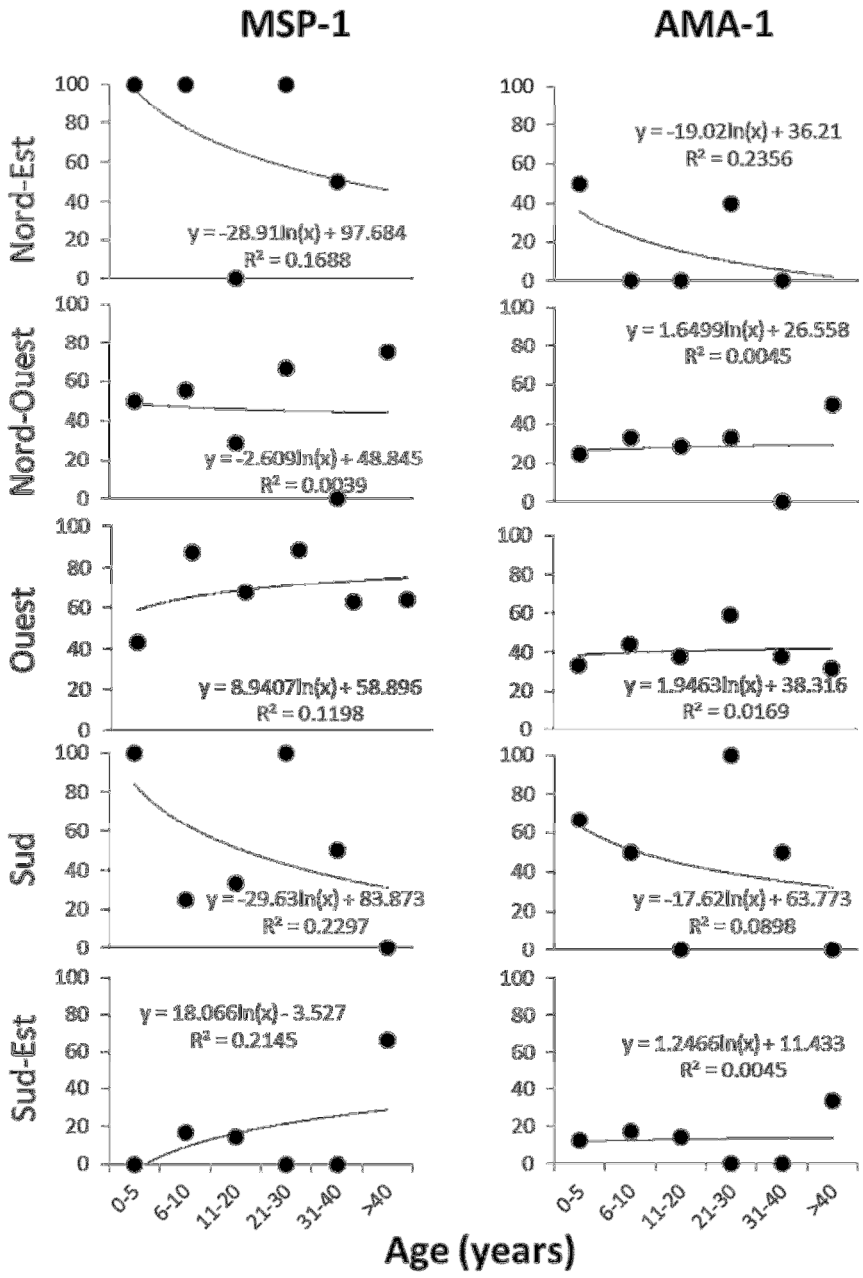


Figure 11, continued. Seroprevalence curves for Haitian departments based on percent population with anti-malarial antibodies. The y-axis displays percentage of the department's seropositive population whereas the x-axis displays age calipers.

Table 2: Summary of Results from Seropositive Curves from Haitian Departments

Department	Sample Number (n)	Estimate of MSP-1 Seroprevalence Rate	MSP-1 Intercept	MSP-1 R^2	Estimate of AMA-1 Seroprevalence Rate	AMA-1 Intercept	AMA-1 R^2
Artibonite	98	0.0597	0.4243	0.043	-0.0077	0.3522	0.003
Centre	58	0.2879	0.2859	0.801	0.3682	0.0842	0.595
Grand'Anse	20	0.4992	0.0637	0.763	0.1022	0.2991	0.039
Nippes	22	0.1331	0.7093	0.301	0.2935	0.0135	0.277
Nord	39	0.3372	0.2601	0.797	0.2445	-0.0384	0.724
Nord-Est	11	-0.2891	0.9768	0.169	-0.1902	0.3621	0.236
Nord-Ouest	28	-0.0261	0.4885	0.004	0.0165	0.2656	0.005
Ouest	170	0.0894	0.589	0.120	0.0195	0.3832	0.017
Sud	17	-0.2963	0.8387	0.230	-0.1762	0.6377	0.090
Sud-Est	28	0.1807	-0.0353	0.215	0.0125	0.1143	0.005
Haiti	**491**	**0.147**	**0.4288**	**0.767**	**0.0829**	**0.2753**	**0.488**

APPENDIX A

2012 Haiti TRaC Survey

Standard Operating Procedure (SOP) to register and split specimens for LNSP and CDC laboratories

Overview: The 2012 Haiti TRaC survey collected approximately 5,500 dried blood spot (DBS) specimens on Protein Saver™ 903® cards and Whatman ® 1 filter paper. Most persons were able to provide five spots, but it was found that fewer than five spots were collected for some specimens.

Molecular analysis will be performed from dried blood spots

- LNSP will conduct PET-PCR analysis on all samples to detect *Plasmodium* infections
- CDC-Atlanta will perform PCR quality control by conducting PET-PCR on a random 10% of the specimens
- CDC-Atlanta will conduct a serological assessment on all samples to evaluate IgG antibody responses from prior exposure to malaria parasites

DNA elution for PCR-based methods of *Plasmodium* detection requires at least one good spot of dried blood. Protein elution for serological assessments requires two good spots.

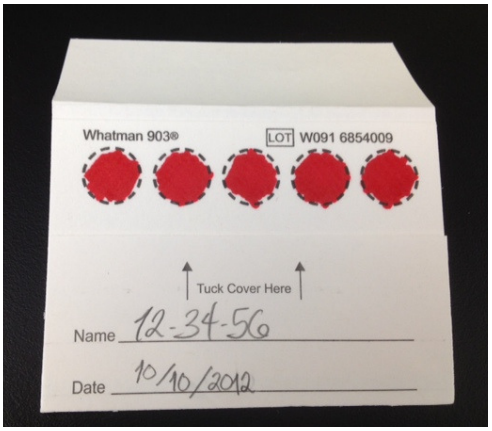
To enable analysis by LNSP and CDC laboratories, the specimens will be split into two portions and the specimen numbers (Subject ID, and Unique ID) will be recorded into a database. This standard operating procedure (SOP) document describes this process.

- During specimen collection, specimens were collected and labeled with a 6-digit **Subject ID**
  (__ __ - __ __ - __ __):
  - First two digits correspond to the enumeration area (also known as SDE number)
  - If 5 digits were provided, it is assumed that the initial digit would have been zero
  - Second two digits correspond to the household number
  - Last two digits correspond to the person number

Upon recording the specimen into the database and splitting the specimens for LNSP and CDC analysis, a **Unique ID** will be assigned to the 5,500 specimens collected. This 4-digit number ranging from 1000–6500 will enable specimens to be tracked back to the original filter paper; in this way split specimens will be linked and corrections (due to

potential errors from poor labeling or mis-typing, etc.) can be made from the original specimen packaging.

Samples on Protein Saver™ 903® booklet



Samples on Whatman® 1 circular (or half-circular) filter paper

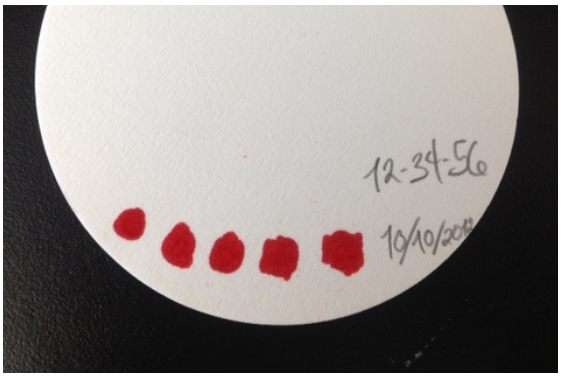


Table 1. Number of Spots

Number of spots collected	Number of spots for LNSP (A)	Number of spots for CDC (B)
5	2	3
4	1	3
3	1	2
2	1	1
1	1	0

Materials required

- Original specimens
- Ziploc bags, clear and Redline®
- 1.5g desiccant packages
- 4-digit Unique ID sticker labels (4 are provided, but only 2 or 3 will be used)
- Clean scissors
- 95-100% ethanol and KimWipes, or ethanol swabs
- Latex or Nitrile Gloves
- Computer with Excel database file

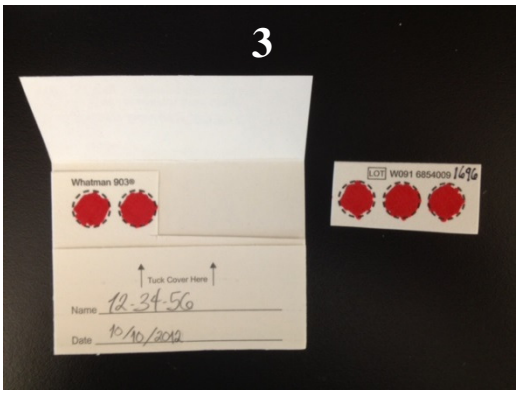
Task	Detailed Steps
1. Set-up space for each worker	a. Each worker should have a clean work space and the following: i. Print off of 6-digit Subject ID for all samples that will be processed that day with columns to add Unique ID, number of spots, and remarks ii. Original DBS specimens (do not need to be in order) iii. Unique ID stickers (do not need to be in order, but sequential numbers will be easiest to record) iv. Clean scissors, 95% ethanol and KimWipes, or ethanol swabs v. New, clear and Redline® bags vi. 1.5g desiccant packages

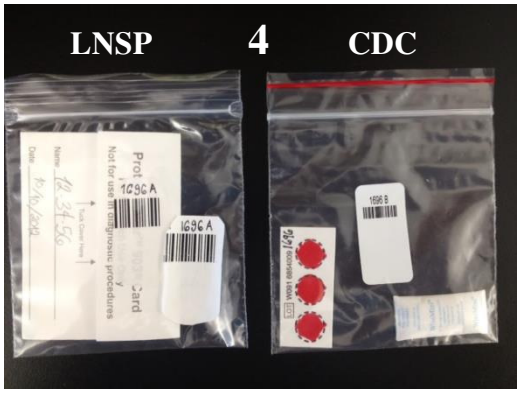
Task	Detailed Steps
2. Labeling and recording	**Throughout the entire processing, efforts should be made to not touch the blood spots with hands or scissors** Two different types of samples will be encountered: 1) Samples in the Protein Saver™ 903® booklet 2) Samples on circular (or half-circular) Whatman® 1 filter paper <u>Samples in the Protein Saver™ 903® booklet</u> For samples on the booklet, three Unique ID stickers will be needed: i) Before taking the booklet from the bag, place one sticker "XXXX A" on the plastic bag containing the booklet. If the booklet is not in a bag, obtain a new clear bag to put the sticker on which will hold the booklet. You do not need to put desiccant in the new clear bag. ii) Remove the booklet from the bag and place one sticker "XXXX A" on the Protein Saver envelope on the outside of the cover flap iii) Place a sticker "XXXX B" on the bag which will hold the CDC sample. <u>Samples on Whatman® 1 circular (or half-circular) filter paper</u> For samples on circular (or half-circular) filter paper, two unique ID stickers will be needed: i) Before taking the filter paper from the bag, place one sticker "XXXX A" on the plastic bag containing the booklet ii) Place a sticker "XXXX B" on the bag which will hold the CDC sample. For all samples, record the 4-digit Unique ID number of the sample in the next column to the right of the 6-digit Subject ID number. Also record the number of blood spots and any additional remarks (most samples will not have remarks): i) Specimen was stored with other samples ii) Specimen was on Whatman™ 1 circular (or half-circular) filter paper iii) Small amount of blood iv) Other **Stickers should be placed before samples are cut!!!**

Task	Detailed Steps
3. Splitting and bagging filter paper	a. Wipe the scissors with 95% ethanol or ethanol wipe to clean them. Refer to "**Table 1. Number of Spots**" to allocate spots appropriately to the LNSP and CDC bags. If all spots are small, make sure LNSP gets the largest spot. b. If the sample is in a booklet, the LNSP spots will stay in the booklet and CDC spots will be cut out. Cut the filter paper making sure to not let the scissors touch the blood spots. c. Using a pen, write the 4-digit Unique ID number on the filter paper for the CDC. If the sample is a circular (or half-circular) filter paper, write the Unique ID number on the split filter papers for both the LNSP and CDC. d. Place the LNSP booklet or spots back in the original bag with the sticker "XXXX A" on the front. Place the CDC spots in the Redline® bag with the sticker "XXXX B" on the front. Also place a 1.5g desiccant in the CDC bag and seal.
4. Storing samples and updating database	a. Keep CDC samples in a box separate from the LNSP samples. Store all samples in cold room at 4°C until ready for DNA purification at LNSP or shipping to the CDC. b. Before leaving for the day, record the 4-digit Unique ID number, number of spots, and any remarks in the Excel database for each 6-digit Sample ID that was processed that day.

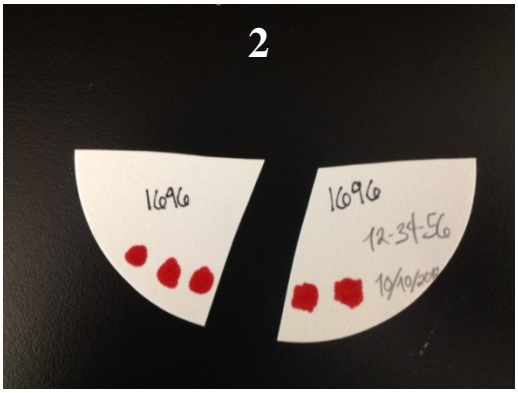
CDC samples placed in a box for shipping

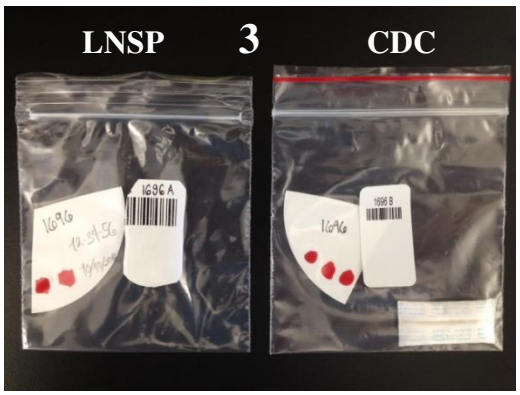
Protein Saver™ 903® booklet





Whatman® 1 Filter Paper





APPENDIX B

2012 Haiti TRaC Survey

Standard Operating Procedure (SOP) for extraction of DNA from dried blood spots (DBS) for LNSP and CDC laboratories

Modified Protocol: DNA Purification from Dried Blood Spots (QIAamp DNA Mini Kit)

This protocol is for purification of total (genomic, mitochondrial, and viral) DNA from blood, both untreated and treated with anticoagulants, which has been spotted and dried on filter paper (Whatman circular and 903 filters).

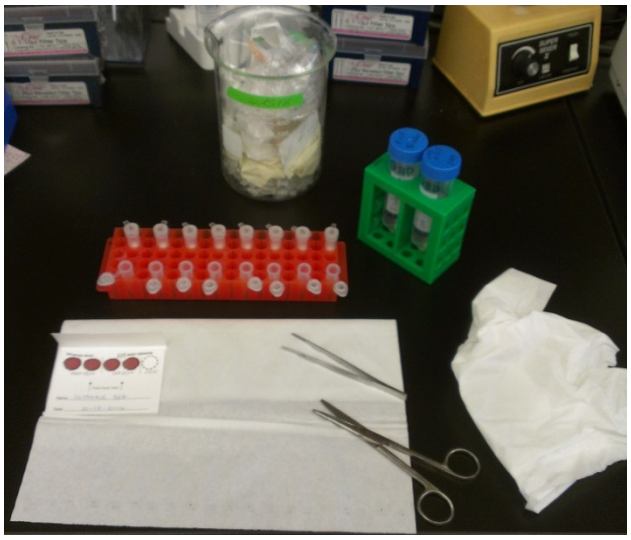
Important point before starting
All centrifugation steps are carried out at room temperature (15–25°C).

Things to do before starting

- Prepare an 85°C water bath for use in step 2, a 56°C water bath for use in step 3, and a 70°C water bath for use in step 4.
- Equilibrate Buffer AE or distilled water to room temperature (15–25°C) for elution in step 10.
- Ensure that Buffer AW1 and Buffer AW2 have been prepared according to the instructions on page 16.
- If a precipitate has formed in Buffer AL or Buffer ATL, dissolve by incubating at 56°C.
- Have scissors, forceps, and cleaning ethanol ready for use

Extraction Procedure

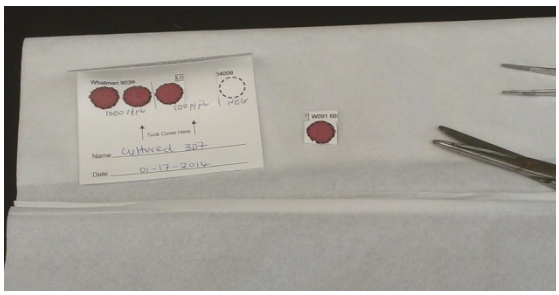
1. Set up work area by placing large Kimwipe or paper towel on bench area to be used for extractions. Also wet a separate Kimwipe or paper towel with ethanol to assist with cleaning between samples.



2. Label 1.5mL microcentrifuge tubes with the unique 4-digit number for the samples you will be processing. It is necessary that you label the microcentrifuge tubes before the extraction procedure begins.

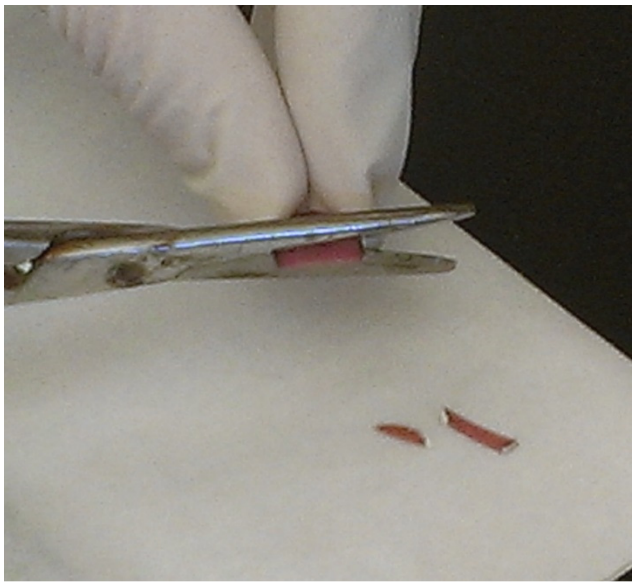
3. Take the Whatman 903 booklet or Circular filter paper from plastic bag, wipe scissors and forceps with ethanol, and cut out one spot for extraction (A). For 903 booklets, try to find the spot most within the dotted lines. Cut only along the dotted lines (B). Form a circle by cutting along dotted line, even if blood does not extend to the line or extends beyond the line. For the circular paper, choose the largest spot.

A)



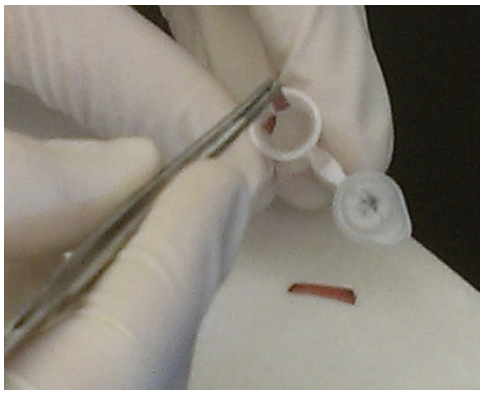
B)

4. Hold the spot in your fingers and cut into 5 horizontal strips, allowing the strips to fall to the Kimwipe/paper towel.

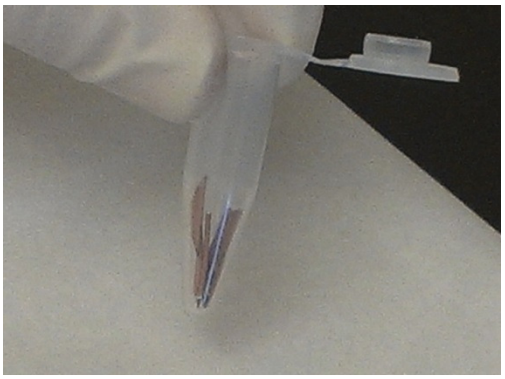


5. Using the forceps, transfer the 5 strips to the microcentrifuge tube with the same
4-digit number (A). Tap the tube on the bench to gather all strips to the bottom of the tube (B). If strips remain on the side of the tube, push them down with the forceps.

A)



B)



6. Add 300 µl of Buffer ATL to the microcentrifuge tube.

7. Incubate at 85°C for 10 minutes. Briefly centrifuge to remove drops from inside the lid.

8. Add 20 µl proteinase K stock solution. Mix by vortexing, and incubate at 56°C for
1 hour. Briefly centrifuge to remove drops from inside the lid.
Note: The addition of proteinase K is essential.

9. Add 200 µl Buffer AL to the sample. Mix thoroughly by vortexing, and incubate at
70°C for 10 minutes. Briefly centrifuge to remove drops from inside the lid.
To ensure efficient lysis, it is essential that the sample and Buffer AL are mixed immediately and thoroughly.
Note: Do not add proteinase K directly to Buffer AL.

10. Add 200 µl ethanol (100%) to the sample, and mix thoroughly by vortexing. Briefly centrifuge to remove drops from inside the lid.
It is essential that the sample and ethanol are mixed thoroughly.

11. Carefully apply the mixture from step 5 to the QIAamp Mini spin column (in a 2 ml
collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x *g* (8000 rpm) for 1 minute. Place the QIAamp Mini spin column in a clean 2 ml collection
tube (provided), and discard the tube containing the filtrate.
Close each QIAamp Mini spin column to avoid aerosol formation during centrifugation.

12. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1 without
wetting the rim. Close the cap and centrifuge at 6000 x *g* (8000 rpm) for 1 minute.

Place the QIAamp Mini spin column in a clean 2 ml collection tube, and discard the collection tube containing the filtrate.

13. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x *g*; 14,000 rpm) for 3 minutes.

14. Place the QIAamp Mini spin column in a new 2 ml collection tube and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 minute.
This step helps to eliminate the chance of possible Buffer AW2 carryover.

15. Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube, and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 100 µl distilled water. Incubate at room temperature (15–25°C) for 5 minutes, and then centrifuge at 6000 x *g* (8000 rpm) for 1 minute. It is very important that the water is able to soak in the column for 5 minutes before the DNA is eluted into the 1.5 ml tube.

16. Store the DNA at -20°C for short-term storage and -80°C for long-term storage.

APPENDIX C

Protocol for Optimization of Malarial Antigens and Haitian Sample Assays for DBS ELISA

Supplies and Liquids Needed

- ThermoScientific Immulon 2HB flat-bottom plate and adhesive plate covers
- Liquid trough and multi-channel pipette
- Automated plate washer
- **PBS**: pH 7.2
- **Wash Solution / Antibody Diluent**: PBS + 0.05% Tween20 pH 7.4
- **Blocking Buffer / Sample Diluent**: wash solution + 5% dehydrated milk
- **TMB Substrate**: A and B bottles ()
- **Stop Solution**: 0.2M H_2SO_4 (Sigma)

Concentration of Reagents

Antigen coating (Step 1): 0.25ug/mL
Sample dilution (Step 3): Final dilution of 1:400x
Secondary HRP conjugate (Step 4): 1:12,000x dilution

Procedure – Refer to systematic template for optimization concentrations

1) Dilute antigen to appropriate concentration in **PBS** and coat plates at 4^o overnight with 100uL/well.

2) Wash plate 3x with **Wash Solution** and add 150uL **Blocking Buffer** to each well. Shake lightly for 2h.

3) Wash plate 3x. Add 100uL/well of samples diluted to appropriate concentration in **Sample Diluent**. Shake lightly for 2h.

4) Wash plate 3x. Dilute secondary antibody in **Antibody Diluent** and add 100uL/well. Shake lightly for 1h.

5) Wash plate 4x. Mix equal parts **TMB Substrate A** and **B** and add 100uL/well. Allow color to develop for 10min.

6) Stop reaction with **Stop Solution** with 100uL/well. Read plates at 450nm on machine using SoftMax Pro software.