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Using Serological Assays Detecting IgG to Estimate Malaria Transmission Intensity in Thies, Senegal

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Abstract

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By Tyler Chavers

Malaria is an international public health issue with over 3 billion of the world's population at risk. Heterogeneous patterns of malaria transmission intensity (MTI) across endemic areas require surveillance systems that estimate malaria exposure at all levels of MTI. Serosurveillance using tools such as Luminex® multiplex assays can provide new estimates of transmission intensity by estimating the strength of antibody responses to infection using median fluorescence intensity (MFI). This study aimed to estimate MTI in the population of Thies, Senegal using a Luminex multiplex assay measuring changes in MFI as surrogates for IgG antibody levels across a longitudinal cohort design. Demographic information and blood spots for IgG multiplex analyses were collected during biannual visits from 2012 to 2015 from a cohort (n = 1,980) in the hypoendemic region of Thies, Senegal. We used these data to calculate novel estimates of MTI, including the annual frequency of seropositives (defined as individuals with MFI values above seropositive cutoffs), the number of annual seropositives with increased MFI values after each transmission season, and to plot individual MFI values for each visit as proxy measures of antibody responses to malaria during the study. There was a 15% increase of seropositives by PfMSP-1_{19} across year 1, -15% in year 2, and -4% in year 3. All other antibody responses displayed a range of -3% to 1% among all species. We identified higher frequencies of seropositives with increases in MFI for *Plasmodium falciparum* throughout each transmission season, with a total 679 for PfMSP-1_{19}, 368 for AMA-1, and 88 for LSA-1 across the duration of the study. Responses to other malarial species occurred at lower levels, with 110 seropositives for PoMSP-1_{19}, 50 seropositives for PmMSP-1_{19}, and six seropositives for PvMSP-1_{19} across all three transmission seasons. Plotting the MFI levels for five individuals at each cohort visit revealed five dynamic trends of malarial exposure across the study, which may provide alternate measures for malaria transmission. These analyses have identified potential methods for estimating MTI using antibody data with success in an area of low transmission, but require testing in other areas and validation before use in malaria surveillance.

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Literature Review

MALARIA

Malaria is one of the oldest and impactful diseases affecting humans. There were over 200 million cases of malaria reported throughout 2015 and approximately 3.4 billion people are at risk worldwide (1). There are four species of *Plasmodium* that cause malaria in humans. *Plasmodium falciparum* results in the most severe form of the disease. It can result in anemia or death in individuals suffering from severe malaria, and can result in low birth weight in babies if mothers are infected during pregnancy (2). Although a rare outcome, *P. vivax* can also result in cases of complicated malaria (3). *Plasmodium ovale* and *P. malariae* comprise the other, less severe types of human malaria. *Plasmodium knowlesi*, a historically zoonotic species, was recently described circulating among humans in the Greater Mekong Subregion and Indonesia (4). Symptoms for uncomplicated malaria include fever, chills, other and several other flu-like symptoms. Severe or complicated malaria is more harmful, and may result in life-threatening conditions such as severe anemia and cerebral malaria (reviewed in (5)).

MALARIA TRANSMISSION

Malaria is a vector-borne disease with heterogeneous transmission patterns throughout the world. It is spread by female mosquitos from 25 of the 400 species in the genus *Anopheles* (6). The malaria parasites reproduce in three distinct life stages, occurring in the midgut of a vector (sporogonic cycle), the liver cells of a human host (exo-erythrocytic cycle or liver stage), and in the blood cells of a human host

(erythrocytic cycle or blood stage) (2). Both *P. vivax* and *P. ovale* have a dormant life-stage (hypnozoites) that can reemerge years later after initial infection. Malaria endemicity within a location can be holoendemic (continuously occurring), mesoendemic (unstable or seasonal transmission), or hypoendemic (sustained transmission at low levels) (7). Areas that sustain holoendemic transmission will see the highest incidence of malaria in children and lower incidence in adults, who have developed long-lasting IgG antibodies due to early, consistent exposure (5). However, meso- or hypoendemic regions often see different incidence patterns. Instead, these regions experience a continuous risk of malaria in adults and children as neither group develops a humoral response against the disease at a young age (8, 9). Meso- and hypoendemic regions are also at highest risk for seasonal changes in malaria transmission, and are more susceptible to malaria outbreaks than other endemicities (10). These heterogeneous patterns of transmission influence who and what regions are affected by malaria, which are imperative for control and elimination efforts. Therefore, malaria surveillance systems must provide comprehensive measures of transmission intensity at all possible endemicities.

MEASUREMENTS OF MALARIA TRANSMISSION INTENSITY

Due to the many dynamics of malaria there several ways to directly measure the number of malarial infections in a defined location over time, known as malaria transmission intensity (MTI). MTI can be estimated using the entomological inoculation rate (EIR), which measures the number of infectious bites per person over a unit of time (11). Parasite rate (or parasite prevalence) and the annual parasite index (API) also

directly measure MTI (reviewed in (12)). Parasite rate is the proportion of a population infected with Plasmodia at a given time, while API is the number of Plasmodia infections per year within an area of interest. The force of infection is another measurement of MTI and is defined as the number of newly acquired Plasmodia infections per unit time (reviewed in (13)). EIR, parasite rate, and API estimates are measured directly using clinical and field diagnostic data, while force of infection is measured using malarial attack rates from cohort studies of susceptible individuals. Force of infection can also be calculated with cross-sectional surveys, where increases in parasite rate across age groups are fitted to reverse catalytic models (14). Each of these direct methods provide useful measures of MTI. However, they suffer limitations including of high costs, training, and decreased accuracy at low transmission intensities (15).

Indirect estimates of MTI have also been made using antibody responses to malarial infection. Antibodies in human hosts can indicate previous exposure to malaria. Serological studies estimate MTI by setting cutoff values for malarial antibody titers, which indicate whether an individual is seropositive (has IgG to antigens) or not. Age-specific seroprevalence data are fit to reversible catalytic models to produce seroconversion rates (SCR) which estimate the probability of malarial seroconversion per year in a specific population (14). The data for SCR come from any assays that detect or quantify antibodies to malaria-specific antigens in human blood. Using serology as an estimate of MTI is advantageous because seroprevalence data can help infer if individuals have been exposed to malaria in the past (15). However, these inferences do not describe the temporality of exposure. Serologic studies have typically focused on cross-sectional surveys with only a few longitudinal studies followed for less

than a year, which have relied on statistical models to explain annual trends (14, 16). These SCR analyses use the binary outcome of malarial seroconversion for their estimates, which simplify changes in immune response and do not take into account continuous changes in antibody titers. Methods investigating individual antibody titer changes over multiple years have not been widely studied to date.

SURVEILLANCE

There are a number of considerations for malaria surveillance. Parasite life stages, heterogeneous transmission, and the diversity of vector and *Plasmodium* species all influence data collection. Other issues, such as emerging resistance to antimalarial drugs (17), emphasize the need for comprehensive surveillance. Several malarial detection methods are currently available. These range from binary infection confirmation tests to sophisticated methods that detect malaria indicators on the molecular level.

Visual Detection

Microscopy

Microscopy has historically been considered the ‘gold standard’ of malaria diagnoses as it allows for the direct observation of Plasmodia in blood (18, 19). Its use began in 1880 when Dr. Alphonse Laveran observed the protozoan in red blood cells under a microscope for the first time to determine them as the causative agent for malaria. Laveran’s initial observations only used a fixed blood sample on a slide sealed

with paraffin (20). The current recommended method consists of collecting a blood sample from a suspected case, preparing a thick or thin smear on a slide, staining it (a Giemsa stain is favored), and examining it under oil immersion at 100X (reviewed in (21)). Thick smears can indicate presence of Plasmodia while stained thin smears have the added benefit of differentiating malaria species, albeit with some difficulty due to similarities in morphology among species (22).

Microscopy has strengths and limitations as a tool for diagnoses and surveillance. Microscopy is relatively inexpensive, can diagnose a case within a day, and allows for enumeration and differentiation of malarial parasites (21). It has a demonstrated 60% sensitivity and 92.5% specificity (19) compared to polymerase chain reaction (PCR) as a reference, but these values will vary based on conditions. In fact, most of microscopy's strengths rely on the staff capacity and infrastructure available. Possible errors include interpreting or reporting slide results as the result of inadequate resources or training (23, 24). Alternatively, visual detection may be unsuccessful for reasons other than technician expertise, and becomes increasingly difficult as the density of parasitemia decreases in a smear and may result in false negatives (25). Also, *P. falciparum* malaria may not be present in peripheral blood depending on its current life cycle (i.e. not in the blood stage), and infections are misdiagnosed due to absence in the smear (26). Furthermore, difficulties in *P. falciparum* and *P. vivax* detection via microscopy have been demonstrated for cases of placental malaria, which are among the highest at-risk populations for the disease (18). Prompt, accurate results are essential in order to treat cases and detect possible drug resistance through non-clearance of parasites, and remains a major limitation for this method (27).

Given its limitations, microscopy is not used extensively in the field in many parts of the world. Labor and time costs encourage monitoring programs to only use microscopy for laboratory diagnosis on suspected cases more often than at point-of-contact (27). Also, it is used as a confirmatory method for cases identified through other field diagnostics that have lower sensitivities and specificities (28). Since microscopy directly detects Plasmodia, it can provide case data for the parasite rate, API, and studies measuring EIR or force of infection. Researchers Roucher et al. demonstrated this utility by using microscopy to observe changes in parasite prevalence for *P. falciparum*, *P. malariae*, and *P. ovale*, in Dielmo village, Senegal over a 20-year period (Figure 1) (29). Although microscopy is the gold standard, recent comparisons to genomic detection methods demonstrate that it is neither the most sensitive nor specific diagnostic for malaria (19, 30, 31).

Molecular Assays

Polymerase Chain Reaction (PCR) Assays

PCR assays for malaria detect specific genomic sequences unique to Plasmodia from infected blood specimens. This is done by extracting genetic material, annealing with primers, and then replicating the genetic material multiple times under oscillating temperatures using a thermocycler (32). The first PCR assays for malaria focused on *P. falciparum* due to its impact, and were developed shortly after the advent of the PCR technique (33, 34). Later assays made it possible to differentiate between all four human strains and *P. knowelsi* by using primers that target species-specific genes in the small subunit RNA (35, 36).

PCR is a strong diagnostic tool, yet has multiple limitations for surveillance. PCR assays that detect ribosomal RNA demonstrate high performance, with sensitivity measured at 100% and specificity at 99.56% using inoculated blood samples as a reference (37). Multiple evaluations have demonstrated that these assays perform well at low parasitemia, are effective at differentiating species, and are more sensitive than microscopy and rapid diagnostic tests (RDTs) (38, 39). Consequently, PCR is often used in research settings as a confirmatory test after initial screenings (28, 40). In addition, its use of genomic targets for detection allows it to monitor genetic diversity in the circulating malaria parasite population (41). However, its detection capabilities come at a price. PCR must be done in a laboratory, is costly, and requires specialized training (42). Researchers have attempted to address these issues by creating mobile platforms for PCR (43). Still, the majority of PCR occurs through stationary labs. As such, cost and infrastructure dictate whether PCR is appropriate for the given setting.

PCR is an infrequent diagnostic used for malaria surveillance due to the required time and resources. Because any PCR method for malaria requires sophisticated equipment they are difficult to adapt to field surveillance. Thus, PCR is often used in research settings rather than clinical settings to detect and differentiate malaria infections within an individual (38, 44). The results from malarial PCR assays provide measurements of acute cases. Identified cases typically contribute to MTI estimates such as parasite prevalence, and more recently for molecular analyses estimating force of infection (15).

Loop-Mediated Isothermal Amplification Assays (LAMP)

Molecular-based LAMP assays amplify genetic material similar to PCR, but require less time and resources. Notomi et al. first described the process in 2000, which uses specific genetic primers to replicate and detect the presence of Plasmodia within blood samples (45). However, LAMP is specifically designed to overcome the infrastructure and training requirements inherent with PCR. Investigators extract DNA from blood samples, which they combine with specific primers and polymerase into a reaction tube. LAMP assays utilize a different polymerase (*Bacillus stearothermophilus, Bst,* polymerase) to compensate for the temperature needs in DNA replication, removing the need for a thermocycler (45). Presence of Plasmodia is indicated by a visible color change in the solution (46) or through software programs that quantify malaria based on the strength of fluorescence (reviewed in (47)). Therefore, the entire process – from extraction to detection – occurs in a single device.

The adaptability of LAMP has both its strengths and limitations. Like PCR, LAMP can diagnose by species of malaria and monitor genetic diversity of circulating malaria populations (48). LAMP tests several samples in approximately one hour at a comparable cost to PCR (47), and is rapidly improving. For example, Perera et al. have recently developed a method for high throughput LAMP assays that test up to 94 samples for malaria per run (49). It has the potential as a useful and portable diagnostic tool, with reported sensitivities at 100% and specificity at 98% compared to PCR (50). However, evaluations have shown LAMP assays may have inconsistencies with detection at low parasitemia levels (51).

LAMP's many strengths allow it to be used in the either the field or laboratory settings. Like PCR, LAMP can serve as a confirmatory test for other diagnostics and as

a tool for genetic surveillance. Yet its most useful quality is as a sensitive diagnostic for point-of-care testing in the field due to its portable design (52). Cases identified by LAMP can provide case data for parasite prevalence, API, and force of infection. Yet, field use for LAMP is limited to individuals with the training and specialization needed for the assay (53).

Serology

Rapid Diagnostic Tests (RDT)

Challenges with timely diagnosis and species differentiation inspired tools that directly detect malaria antigens outside of a laboratory known as rapid diagnostic tests (RDTs). In the early 1990s Shiff, Premji, and Minjas described the first RDTs that could diagnose malaria infection at a fraction of the time for microscopy while only using a drop of blood. The first commercially available RDT was *Para*Sight®-F, a test strip that indicated the presence of captured proteins and antigens produced by malarial parasites circulating in the body (54). Modern tests utilize handheld cassettes that contain an antibody or multiple antibodies dried on a filter paper test strip that corresponds to malarial antigens. A drop of blood is placed in a collection well that functions using lateral flow immunochromatography (55). If present, *Plasmodium* antigens from blood will bind to capture and detection antibodies to form a band indicating positivity in roughly 15 minutes. These cassettes are customizable with broad or species-specific agents for detection (56). Currently, the WHO estimates that there are over 200 distinct RDTs for malaria on the market, many of which detect the same protein targets (57).

RDTs are a unique tool for surveillance with a number of advantages and limitations. RDTs are useful because they provide a quick and accurate diagnosis with minimal training. This feature compensates for the logistical limitations of laboratory methods and is an alternative when microscopy is not feasible (28). RDTs also perform relatively well in field evaluations with a sensitivity range of 71.1-75.4% and specificity range of 80.8-84.8% compared to microscopy as a reference (28). Consequently, RDTs are recommended by the WHO and have become the most widely used method for point-of-care malaria diagnostics to ensure quick diagnosis and proper treatment (57). Many RDTs detect the histidine-rich protein 2 (HRP-2) as it persists in the human host after malarial antigens are cleared (58). However, the emergence of HRP-2 deletions in the Amazon region of South America has the potential to make HRP-2 tests ineffective (59). Other limitations of RDT performance can depend on factors such as storage temperature, handling, and parasitemia levels in the host (60).

RDTs have become an integral part of malaria programs worldwide. The recent WHO Global Technical Strategy for Malaria 2016-2030 emphasizes the role RDTs serve today. In the comprehensive plan, the WHO Global Technical Strategy for Malaria 2016-2030 stated that using RDTs is an important step towards universal testing of all suspected malaria cases for quick diagnosis and treatment (61). RDTs also have implications at population levels. Cases identified by RDTs have been used to monitor malaria morbidity in endemic regions of Africa (62), South America (63), and Asia (64). In fact, many countries have incorporated RDT testing as an integral part within their control and elimination programs (28, 62). These identified cases assess MTI by providing data for parasite rate and API estimates. However, inconsistent sensitivities

and limited species differentiation of RDTs often require confirmation through microscopy or PCR tests (65). Nevertheless, RDTs will likely remain necessary for malaria control and elimination efforts globally, as they are more portable and require less specialization than microscopy or genetic amplification methods.

Indirect Fluorescent Antibody and Enzyme Immunoassays

Several laboratory tests can indicate if a person has ever been infected with malaria by detecting antibodies in their blood. Antibody detection is not useful for acute diagnosis, but can provide information on previous exposures. One of the first methods to detect malarial antibodies in a human host was the indirect-fluorescent antibody (IFA) test (66). IFA detection for malarial antibodies begins with incubating parasitized red blood cells with patient serum on a microscope slide. If present, malaria-specific antibodies in the serum will form an antigen-antibody (Ag-Ab) complex. Next, a fluorescent-dyed anti-human immunoglobulin is added to tag the Ag-Ab complexes so they can be observed under a fluorescence microscope (67). Shortly after, enzyme-based immunoassays (EIA) were developed for malaria. The EIA is a class of assays that use enzyme-labeled antigens or antibodies to detect malarial antibodies (using antigens) or antigens (using antibodies) in human hosts. Enzyme-linked immunosorbent assays (ELISA), a type of EIA, are commonly used for malarial antibody detection. ELISAs adapted for malarial antibody detection use a substrate containing enzymes linked to immobilized malarial antigens held within test wells. The prepared wells are then incubated with serum, and if malaria-specific antibodies are present they will adsorb to the surface of the substrate. Anti-human immunoglobulins linked to enzymes

are added to the solution to mark adsorbed antibodies. The well is then incubated with an enzyme substrate to react with the enzymes conjugated to the antibodies to produce a visible change in the substrate (i.e. color change). Analyses then use spectrophotometers to read the optical density (OD) of the substrate to quantify titers (68). Success of the assay can vary across ELISAs and are influenced by the antigens used and specific malaria species prevalence (69, 70). Today, IFA and EIA antibody detection are available for all human malarial species and *P. knowlesi*.

Measuring the response to infection rather than the presence of infection allows for unique analyses of malaria transmission. According to Bretscher et al., because malarial antibodies last longer than an infection, they leave a 'footprint' indicating if someone was ever exposed to malaria (16). They state this can be useful in areas with low transmission where detection is more difficult. The authors quantified monthly antibody titers (estimated by OD) of school children for six months. They used titers from a malaria naïve population to establish a threshold (mean + three standard deviations), and classified anyone above this threshold as seropositive. They argued that obtaining a SCR by fitting malarial seroconversion data to a model provided a correlate of force of infection. Still, the 'footprint' revealed by ELISA did not indicate the temporality of exposure or seroconversion. Therefore, this method is not recommended as a tool for individual diagnosis (67).

Antibody tests are typically used for malaria surveillance on a population level rather than an individual basis. Both IFA and ELISA are routinely used to screen blood donations for malaria (69, 71, 72). They are also effective tools to quickly measure malarial exposure in a population (73). Still, field applications of IFA and EIA remain

limited to cross-sectional or brief longitudinal surveys, and usually provide SCR data to estimate MTI.

Luminex® Multiplex Assays

Multiplex assays expand upon single antibody tests like EIA to generate large amounts of serologic data. These multiplex assays work similarly to other antibody-based methods and have demonstrated similar sensitivities and specificities (74). However, multiplex assays can detect multiple, specific targets simultaneously. The Luminex® assay is a type of multiplex assay increasingly being used in malaria serosurveillance. Like the ELISA, this test utilizes a 96-well template. However, each well contains magnetic microspheres coated in malaria antigens that can detect up to 100 targets per well (75). These microspheres each have a unique spectral signal that will fluoresce after binding to a target (76). Multiplex assays have demonstrated the ability to detect multiple species-specific antibodies (77), cytokines (78), and nucleic acids (76) from a single blood spot. The development of this assay has generated new possibilities in multiple diagnostics across different fields. However, the use of this technology for malaria has yet to be fully realized.

Few Luminex studies exist involving human malaria, and most studies occurring within the last several years have focused on testing its potential estimating MTI. Initial reports established Luminex as a rapid, high-throughput genotyping technique for infectious disease surveillance. Researchers Horton et al. described one of the first uses towards malaria surveillance by using Luminex to observe genetic variability in single-nucleotide polymorphisms (SNPs) that may influence uptake of malaria in

mosquitos (79). The investigators genotyped a sample of *Anopheles gambiae* from Mali using the Luminex platform to detect variations in nucleic acid sequences among 11 SNP loci simultaneously. The results of this assay indicated that there were three genetically distinct variants of the vector circulating in the area with differing potential to spread the disease. The researchers verified Luminex quality by comparing the multiplex results of a subset of 48 *An. gambiae* to direct sequencing by PCR, which displayed over 98% agreement.

The ability of Luminex to analyze multiple targets quickly and at high sensitivity revealed its potential as a dependable tool in disease surveillance. In 2012, Lammie et al. described this potential. The authors noted that integration of bead-based multiplex assays into malaria and other disease programs could facilitate surveillance and mapping of multiple diseases, due to its ability to analyze multiple, unique targets simultaneously (75). Researchers Perraut et al. were some of the first to describe the application of this process. In 2014, they used the MAGPIX® Luminex assay to analyze two distinct antibody responses to *P. falciparum* (PF13 and PfMSP-1_{19}) from a sample population in Ndiop, Senegal. Antibody responses in this meso-endemic area were characterized by Luminex median fluorescence intensity (MFI) values produced by antibody-bound beads formed during the assay and ELISA OD values. The MFI values measured in the sample population were compared to MFI results from a negative control group from France to determine seropositivity. Researchers assumed members of the negative control group had never been exposed to malaria, thus MFI values obtained from their blood would determine an appropriate background signal of those never exposed to malaria. Positive antibody responses from the Ndiop sample were

defined as MFI values greater than two standard deviations above the mean of the negative control group (74).

The use of MFI to quantify antibody levels in a population became a staple for successive multiplex studies internationally. In 2015, Koffi et al. determined antibody responses of symptomatic malaria cases by comparing MFI levels compared to a non-immune, foreign population (threshold of mean + three standard deviations) in endemic regions of Ivory Coast (80). This study included three immune response biomarkers: PF13, PfMSP-4_{20}, and PfMSP-1_{19}. That same year, Rogier et al. described transmission of malaria in Haiti using Luminex assays. The impetus for this study was to understand malaria dynamics in a low transmission region, as conventional surveillance methods might not detect these cases (81). This study expanded the use of bead-based multiplexes by quantifying antibodies for the MSP-1_{19}, MSP-1_{42}(D), MSP-1_{42}(F), and AMA-1 antigens, and furthered work on the threshold method. Again, MFI values estimated antibody response and used a naïve population (US) to determine a threshold for seropositivity. This study found that a lognormal distribution of MFI values fit best, and used this transformation to determine the threshold values. They compared two thresholds at three and five standard deviations above the lognormal means and compared the frequency of seropositivity between the two metrics. They found that there were no significant differences between the two threshold methods and that the three standard deviations above the mean cutoff was appropriate (81).

Later studies by the CDC and international researchers have continued to verify new antigens for use in Luminex assays to expand the test's utility. For example, including multiple species-specific antigens for malaria enhances surveillance for

regions that are co-endemic. This was demonstrated in the CDC study by Rogier et al., which included antigens for *P. falciparum* and *P. vivax* in Haiti (81). A later study in Cambodia led by the University of Antwerp, Belgium tested other serological markers, including *Plasmodium*–specific peptides and recombinant proteins (82). Further validation of markers that accurately estimate malaria transmission must be addressed to improve the strength of transmission intensity estimates from Luminex assays.

Recent studies have also used Luminex assays to describe malaria across geographical patterns. In 2016, Kerkhof et al. described the process of using a Luminex assay to understand geographic distribution of malaria in Cambodia compared to PCR prevalence and passive case detection (PCD) estimates. They described potential hotspots of sustained malaria transmission prolonged due to asymptomatic carriers. Thus, PCD in the region was supplemented with PCR surveillance to detect all cases. They argued that serology assays using five antigens ($PfGLURP_{R2}$, $PfMSP\text{-}1_{19}$, and CSP for *P. falciparum*; PvAMA-1 and $PvMSP\text{-}1_{19}$ for *P. vivax*) could detect more malaria hotspots than combined PCR-PCD, using estimates from cross sectional studies in 2012 and 2013. The authors randomly selected 3264 (2012) and 3238 (2013) blood samples from the studies for Luminex analysis using natural log-transformed MFI values. The serology results identified new, smaller, and larger geographical patterns for hotspots compared to previous estimates (83). Interestingly, the distribution of *P. falciparum* varied based on the type of antibody target (Figure 2). This was one of the first studies to describe geographic patterns, which has potential for expansion in future multiplex studies.

Prior studies have demonstrated Luminex's effectiveness for malaria surveillance, as well as highlighted its strengths and limitations. Like other serologic methods, Luminex is a quantitative immunoassay. These data have an inherent degree of background signal "noise" and may fluctuate due to coinfections or random measurement error during analysis (16). The assay also requires highly specialized training to perform. Furthermore, Luminex multiplex approaches are relatively new, and there is little experience in methods, analysis, and interpretation. However, the assay's performance is comparable to established serologic methods such as ELISA (74), enabling it to measure MTI through SCR and parasite prevalence estimates. In addition, its ability to detect and quantify numerous target antibodies simultaneously makes it a powerful tool for integrated disease surveillance.

STUDY AREA

Senegal has been a focus for malaria serosurveillance conducted by the CDC using Luminex assays. The described cohort study was conducted in the city of Thies, located near the western coast of Senegal with a population of over 1,900,000 (84). Thies is located approximately 70 kilometers from Dakar and has tropical climate. It experiences hypoendemic malaria transmission influenced by a high-transmission (rainy) season in the fall (65). In 2006, the country began a new malaria control strategy, which reduced malaria transmission through vector control and case management using RDTs (85). Programs use RDTs extensively in the region for case detection and surveillance (86, 87), but these do not differentiate other species from *P.*

falciparum (65). This is a potential barrier for surveillance as *P. ovale, P. malariae*, (29) and *P. vivax* (88) circulate in the area.

NEEDS, GOALS, AND AIMS

As Thies, Senegal reduces its burden of malaria, it will require comprehensive surveillance and accurate measures of MTI for all circulating species. The use of conventional tools such as microscopy and RDTs to estimate transmission intensity is useful for suspected cases, but may miss transmission occurring at lower parasite levels. There is a **need** to understand malaria transmission among the entire population of Thies, using serological data to display antibody dynamics over time. The **goal** of this study is to estimate malaria transmission intensity in the population of Thies, Senegal using a Luminex assay measuring changes in IgG antibody levels across cohort visits.

Aims

- To describe distribution and changes of malaria antibody titers in pre- and post-transmission seasons within the population of Thies, Senegal
- To calculate transmission intensity through incidence of seroconversion among individuals by applying a seropositivity threshold to individuals within the population

SIGNIFICANCE

Using Luminex to analyze and interpret changes in antibody titers over time in a longitudinal cohort from the population of Thies, Senegal will measure transmission intensity in a novel way. These results aim to indicate changes in malarial

seroconversions and describe individual trends in antibody titers across multiple years. Observing these trends will enhance understanding of malaria transmission in Thies across high-transmission seasons. Furthermore, serosurveillance can provide a more robust picture of transmission in the population compared to antigen detection assays by focusing on the response to infection rather than the presence of antigens. Luminex multiplex assays additionally have the potential to provide more data on circulating malaria species than other surveillance methods currently used (i.e. microscopy and RDTs). Thus, including serology multiplex assays into surveillance systems will guide future responses and interventions with more precision than before.

Methods

SAMPLE POPULATION AND LUMINEX ASSAY

A full description of the cohort study protocol can be found in Appendix B. Briefly, the Senegalese National Malaria Control Program (NMCP) conducted a prospective cohort study involving seven visits in Thies, Senegal from 2012-2015 (Table 1) to investigate malaria transmission and epidemiology in the region. Study surveys were approved by the University Cheikh Anta Diop IRB and non-engagement status in human subjects research was approved by CDC IRB. At cohort visits, study staff performed demographic surveys, provided malaria testing by microscopy and RDT, and collected blood spots (approximately 60µl) on filter paper for analysis using Luminex multiplex assays. The Luminex assays detected IgG antibody responses to the 19kD fragment of Merozoite Surface Protein11 for *Plasmodium falciparum* (PfMSP-1_{19}), *P. ovale* (PoMSP-1_{19}), *P. malariae* (PmMSP-1_{19}), and *P. vivax* (PvMSP-1_{19}), as well as Apical Membrane Antigen-1 (AMA-1) and Liver Stage Antigen-1 (LSA-1) for *P. falciparum*, and were quantified by MFI values. The assays included control wells to detect background MFI values, which were subtracted from measured levels to produce final MFI-bg measures for each antibody response. Study staff also obtained blood samples from a US community blood bank (n = 92) assumed to have no exposure to malaria to run in the Luminex process to determine seropositivity cutoffs. Individuals whose MFI-bg levels were three standard deviations above the mean of the US cohort were considered seropositive for the corresponding IgG. The MFI-bg cutoffs were measured at 261 for AMA-1, 204 for LSA-1, 128 for PfMSP-1_{19}, 379 for PmMSP-1_{19}, 270 for PoMSP-1_{19}, and 581 for PvMSP-1_{19}. Demographic and serology data were compiled into separate excel

databases throughout the study.

DATABASES

The database containing demographic survey data included information for all visits that participants attended. Cohort information included a specific identifier for each participant, the corresponding visit number, visit date, date of birth, sex, age, age group (see Table 2), temperature, weight, if they reported having a fever in the last 24 hours, hemoglobin levels, and classification of anemia severity. This database also contained malaria diagnostic results, including malaria blood smear results from two separate microscopists, the amount of asexual parasitemia per microliter of blood, the gametocyte density per microliter of blood, results from RDTs performed at study visits, the MFI-bg values for IgG responses to MSP-1_{19}, AMA-1, and LSA-1, and whether they were considered seropositive by IgG response based on seropositivity cutoffs from the malaria-naïve US population previously described.

DATA ORGANIZATION

All previously described cohort information was compiled into a single Microsoft Excel database. Each row represented the data collected during visits, and contained values for each participant and the visits they attended. Serology data generated by Luminex assays was stored separately and had to be merged with the cohort database corresponding to the participant and visit number. There were no unique ID numbers linked to the serology database. However, each sample's ID number was written on a paper 96-well template by the laboratory technician (Figure A1: Appendix A), which

indicated the visit the samples corresponded to. The serology database was organized in order of the wells assayed by the technician.

To merge both survey and serology data, the cohort database had to be separated by visit number and organized in the order the samples were assayed by Luminex. Separate databases were created for each visit from the cohort database (i.e. all information for visit three was moved into a new database). Each plate was transcribed into Microsoft Excel templates (Figure A2: Appendix A) with variables ID_Number to match the cohort dataset and Input_Order to organize by the order they were assayed. The populated templates were imported into SAS as new datasets and sorted by ID_number (Appendix C). Next they were merged with each visit's cohort data and sorted by Input_Order. Since the entire datasets for the visits were merged during this process, all variables that were not part of the current plate (i.e. had no value for Input_Order) were deleted. The final datasets were exported to Excel files and combined based on visit number. Once organized, the cohort datasets for each visit were merged with the existing serology databases.

DATA ANALYSIS

Demographics

Demographic and medical information were summarized using survey information from the cohort for each visit, including sex, age, anemia status, fever reported in the last 24 hours, and malaria cases identified by microscopy and RDT. Individuals missing data from any of these categories were omitted from analysis.

Distribution of All Cohort lnMFI-bg Values

The distribution of MFI-bg values obtained throughout the study for each IgG target were assessed for normality and skewness. The frequencies of MFI-bg values were plotted using the PROC UNIVARIATE procedure in SAS v9.4 (SAS Institute Inc., Cary, NC) followed by a HISTOGRAM statement separately for each IgG target. Initial distributions of MFI-bg values showed positive skewing for IgG against different antigens, which has been demonstrated before in areas of low malaria transmission (81). Therefore, MFI-bg values were natural-log (ln) transformed from each visit to facilitate analysis. Any negative MFI-bg values calculated during Luminex analyses were recoded as one (ln = 0), under the assumption that the negative MFI-bg values were indicative of no immune response The lnMFI-bg values were plotted using the HISTOGRAM statement, and the lognormal-transformed seropositivity cutoffs were overlaid to demonstrate the range of seropositives and seronegatives for each IgG target.

Incidence of Seropositives

We measured the number of new seropositive cases by each IgG target at every visit as a potential measure of MTI. For this analysis we counted the number of individuals at each visit whose MFI-bg values were above the seropositive cutoff values from the malaria-naïve US population. The number of seropositives was divided by the n for each corresponding visit to obtain a percent of increases in seropositivity per visit. A percent change in the proportion of seropositives was determined by subtracting the pre-transmission season percentage from the corresponding post-transmission season

percentage for each year of the study. Selection criteria for this analysis considered those with complete data for all IgG, thus the denominators for this analysis differ from those in the demographic summary.

Incidence of Positive Changes in MFI-bg Above Seropositive Threshold

We used the incidence of individuals with positive increases in MFI-bg values throughout the high-transmission season above seropositivity cutoffs as another estimate of MTI. This specifically involved individuals who had paired visits (attended both pre- and post-transmission visits for a given year) for each year of the study to determine the overall change in MFI-bg levels among all six IgG targets. Visit 7 was omitted as there was no corresponding post-transmission visit for that year. Any positive increase was considered for incidence regardless of the magnitude of change, under the assumption that any change in the positive direction is a result of immune response to malaria exposure.

Distribution of Changes in lnMFI-bg for All Paired Visits

The distributions of the changes in MFI-bg among paired visits were observed using the PROC SGPLOT procedure in SAS to obtain boxplots of all differences by IgG. Each MFI-bg value was lognormal-transformed prior to calculating the difference between visits in order to facilitate analysis. The displayed differences in lnMFI-bg were also stratified by IgG type and their classification as seropositive or seronegative.

Individual Trends in MFI-bg Values Across Visits

The availability of MFI-bg values at cohort visits for each individual enrolled in the study has enabled a possible MTI measure by observing longitudinal trends in individual immune responses. To demonstrate the potential utility of this, we selected individuals who had MFI-bg information for each IgG type at all visits. The MFI-bg values for PfMSP-1_{19} at each of the seven visits were plotted for five individuals in order to interpret how longitudinal changes in MFI-bg may estimate variable exposure (and possibly MTI) over time. Five individuals were selected from a pool of 320 individuals with data for each visit in order to display dynamics in antibody responses using this concept while maintaining interpretability of the plots.

Results

Thies Population Characteristics and Dynamics

The described cohort study collected demographic and serological data for 1,980 individuals living in Thies, Senegal. Study staff requested that participants provide a blood spot for analysis of serological responses biannually, before the transmission season in August and directly after the transmission season in February, from Fall 2012 to Fall 2015 (n = 7 visits). Enrollment occurred in Thies during the first three visits, but new enrollment was not continued after these initial periods. Persons not providing a blood sample for one of the visits were still followed-up at each subsequent visit and requested to participate. The distribution of sex and age among the study population remained consistent across each visit (Table 2). Loss to follow-up occurred at varying levels between visits. The lowest participation in demographic surveys occurred in three out of the four pre-transmission season visits (Table 2). The mean proportion of the population experiencing any form of anemia defined by WHO criteria (89) (See Fig. B1: Appendix B) was 42.5% across the first six study visits (no data collected during Visit 7). Thies demonstrates a lower average of anemia prevalence compared to the rest of Senegal, which averaged 76% among children 6-59 months, and 54% among women 15-49 years (90). Undifferentiated fever, a common symptom of malaria (22) and other infectious diseases, and malaria cases identified by microscopy and RDT were found in low percentages of the population at the time of the survey (Table 2).

Serology Results

Analysis of blood spots provided by cohort members estimated changes in antibody titers measured by MFI-bg values from Luminex assays. The number of individuals who provided blood spots differed slightly from those who provided complete demographic surveys, and thus the n values for Table 2 and Table 3 differ at corresponding visits. After log transformation, the PfMSP-1_{19} and AMA-1 distributions were right-skewed, while LSA-1 for *P. falciparum* and the MSP-1_{19} for the three other malaria species displayed normal distributions (Figure 3). The seropositive cutoffs obtained by Luminex results from a United States cohort (see Methods) were displayed on the lnMFI-bg histograms. Inspection of this showed that the selected US population provides cutoffs near the end of the right tail for all IgG types as expected in a region of low MTI due to low incidence. These data also show that the classifications as seropositive for on AMA-1 and PfMSP-1_{19} contain a higher proportion positives compared to the other IgG types, and that each antigen for *P. falciparum* yielded different frequencies of seropositives.

Subsequent analyses applied the pre-determined seropositive cutoffs to changes in for MFI-bg to estimate frequency of exposure over transmission seasons. The yearly percent changes of cohort members whose MFI-bg values increased above cutoff values from pre- to post-transmission seasons are displayed in Table 3. These results show the largest changes of seropositive status occurred in PfMSP-1_{19}, in both positive and negative directions. All changes in seropositivity for the remaining five IgG types varied close to zero across each year. Further analyses on antibody responses to

malaria used the total number of increases in MFI-bg whose post-season value was measured above the seropositive cutoff. This provided a proxy measure of incidence assuming that antibody titers would only increase due to malaria exposure. Again, PfMSP-1_{19} showed the most noticeable trend with the highest frequency of increased MFI-bg values above the cutoff compared to all other IgG types (Figure 4). *Plasmodium ovale*, *malariae*, and *vivax* were all detected but at lower levels. The AMA-1 and LSA-1 targets also detected *P. falciparum* exposure in the Thies cohort (Figure 5), but at lower levels than the corresponding MSP-1_{19} target (Figure 4).

Observing changes in antibody titers across malarial transmission seasons has not been extensively studied. In order to examine these changes in the described cohort, we created boxplots of all lognormal-transformed MFI-bg differences detected using Luminex between paired visits and stratified them by seropositivity classification (Figure 6). The broadest distribution among those classified as seropositive occurred in PfMSP-1_{19} and AMA-1, with values ranging from zero and five (AMA-1) and eight (PfMSP-1_{19}) on the log scale. All other seropositives displayed shorter distributions with ranges below five on the log scale. Among those classified as seronegative, PfMSP-1_{19} and AMA-1 again demonstrated the largest ranges of differences based on outliers ranging from three to negative six (AMA-1) and negative eight (PfMSP-1_{19}). Yet, their corresponding IQRs displayed comparable ranges to the other IgG responses, similar to the seropositive results.

Individual Immune Response Profiles

Generating antibody titer estimates using a longitudinal sampling design provided the opportunity to observe antibody dynamics to malaria antigens for individuals with multiple time points. This was explored with the data from the Thies cohort by plotting the MFI-bg levels measured at each visit for five individuals that attended all seven visits. PfMSP-1_{19} was used as it generated the greatest immunologic response in individuals throughout the study. Again, this assumes that increases in the selected PfMSP-1_{19}–specific IgG only occur due to malarial exposure. Figure 7 displays the MFI-bg profiles of the five selected cohort participants to demonstrate these unique trends.

Discussion

The described study takes a novel approach to measuring MTI by using longitudinal antibody data. Previous studies have used cross sectional or brief longitudinal data on antibody responses to fit statistical models that estimate risk of malaria in the population (14, 16). These approaches rely on indirect estimates rather than observing direct trends, and generalize MTI as homogenous throughout an entire year. Directly observing immune responses to malaria across high transmission periods (i.e. rainy seasons) may provide more precise and appropriate estimates of MTI. This is increasingly important as countries decrease malaria transmission through control efforts.

Within Senegal, malaria transmission has been declining which may be due to efforts such as the NMCP implemented in 2006. The reduction of disease in Thies may be evidenced by the low prevalence of anemia, which may be unaffected by malaria transmission, as well as the few number of cases detected during study visits (Table 2). Active surveillance has detected similar declines of malaria across Senegal by using RDTs and labor-intensive microscopy to surveil populations (29, 87). However, both anemia and case detection may be confounded by factors such as improved nutrition or timing of the diagnostics. Thus, these complexities exemplify the difficulties of malaria surveillance as a country reduces its burden of malaria. We attempted to address these obstacles by describing multiple novel methods of using malarial antibody responses to estimate MTI over time. These involved observing percent change in seropositives over transmission seasons, quantifying the frequency and percent of individuals who

increase in MFI-bg levels over transmission seasons, and displaying dynamics of IgG titers estimated by MFI-bg across seven study visits. Comparison of these novel methods to established measures of MTI provide insight to their accuracy and utility.

In the current study, we evaluated several novel methods to estimate MTI using these changes in malarial antibodies over time on an individual level through a Luminex multiplex assay. Using seropositivity cutoffs as previous studies have described (81), we calculated the incidence of individuals converting to seropositive at each visit of the study. The resulting differences of conversions between pre- and post-transmission seasons produced estimates of MTI for those intervals shown in Table 3. The changes in seroconversion show similar trends to the cases of malaria identified during study visits (Table 2). The highest percent change in seroconversions occurred in between visits 1 and 2, while the largest amount of cases identified by microscopy and second largest among RDTs also occurred between those same visits. It appears that the amount of cases identified by microscopy and RDT may be related to MTI estimates produced by this method. Furthermore, the observed differences for PfMSP-1_{19} showed varying levels of incidence (both positive and negative), while other IgG types showed little to no deviation from zero. It is interesting that other IgG targets for *Plasmodium falciparum* did not provide much variation compared to PfMSP-1_{19}. This phenomenon may be explained by greater levels of affinity for antibodies to MSP-1_{19} compared to other epitopes and antigens (91), or the robust immune responses generated by MSP-1 antigens in humans that has made it a candidate for malaria vaccines (92). Figure 2 demonstrates these as possible explanations as the distribution of PfMSP-1_{19} contains the largest proportion of responses at high lnMFI-bg levels. Additionally, the right-

skewed distributions for AMA-1 and PfMSP-1_{19} antigens represented in Figure 3 demonstrate that this population experiences exposure to *P. falciparum* more than other species. The less-skewed distribution for LSA-1 for the same species may suggest that the immune response to LSA-1 may not produce a lasting response. Additionally, it appears not as sensitive to detecting malaria exposures compared to other antigens, but could be useful in serosurveillance as a specific antigen to confirm when someone has not been exposed to the disease.

The current study also estimated MTI by counting the frequency of individuals among all paired samples with any increases in MFI-bg values that pass the seroconversion cutoffs, assuming any positive change is due to malaria exposure (Figures 4 and 5). The results provided comparable trends in species and magnitudes to the parasite prevalences determined by previous studies. Specifically, this analysis identified *P. falciparum* as the most frequent malarial exposure in Thies, with *P. ovale*, *P. malariae*, and *P. vivax* contributing only a fraction of the burden. These same trends in species and prevalence have been observed in Senegal using the gold standard of microscopy (Figure 1) (29). Niang et al. also described similar trends of *P. falciparum* transmission in Senegal using PCR, but did not detect any *P. malariae* or *P. ovale* (88). Surveillance using RDTs identified 12 (6.4%) symptomatic cases of *P. falciparum* in a region near Thies, but did not identify other malarial species due to the type of RDT used (87).

Quantifying individuals with increases in IgG through the MFI-bg signal has detected both higher proportions and more species of malaria compared to these other

surveillance techniques, which may be due to the extended timeframe of malaria exposure captured by serology or more seropositives identified at lower parasitemia levels using serology compared to antigen detection methods. However, these comparisons assume MTI is homogenous across Senegal. Among the three responses evaluated for *P. falciparum* antigens, MSP-1_{19} IgG displayed the greatest incidence of seropositivity, followed by AMA-1 and then LSA-1. This is slightly contradictory to results obtained by Drakeley et al., who observed the largest seroprevalence in AMA-1 followed by MSP-1_{19} using ELISA OD values from cross-sectional surveys in Tanzania (14). A limitation for this method is that it may be overly sensitive as it includes any positive change in the estimates. As previously mentioned, Luminex assays inherently experience noise, which may result in increases for fluorescence intensity readings without exposure. However, we attempted to compensate for this by subtracting out background noise to create MFI-bg values and by only using those classified as seropositive. Also, humoral responses to malaria may build slowly or fully develop after multiple exposures (93). Additionally, more individuals would naturally experience a decrease in lnMFI-bg rather than an increase due to loss of memory B cells without continued antigenic exposure (94), which further supports the assumption that a positive increase is a good estimator for malarial exposure. Therefore, it is necessary to use this assumption to capture all potential IgG responses to exposure at lower magnitudes.

Luminex was further assessed by observing the distribution of all differences produced during the study (Figure 6). Among those considered seropositive, the majority of differences occurred at low magnitudes. It is likely that many of these individuals already contained anti-malarial antibodies and therefore were either above

the threshold or did not require a large change in MFI-bg to move above the threshold. Among the IgG types for *P. falciparum*, PfMSP-1_{19} detected differences at larger spreads and at higher magnitudes, followed by AMA-1. This is likely due to the consistent responses generated to MSP-1 antigens that have been previously demonstrated (95). It also demonstrates that the Luminex assay using the MSP-1 antigen can detect the highest range of differences in antibody titers. Those classified as seronegative displayed large proportions of individuals at or around zero difference in MFI-bg across transmission seasons. These may be indications of true non-exposure to malaria, as no difference in MFI-bg is expected in the absence of exposure and any MFI-bg values would be considered to be noise of the assay. However, there are some values above zero among those classified as seronegative. This may be due to machine noise or reading error, or from infrequent exposure to malaria which has shown to elicit smaller immune responses compared to repeated exposure (93). Additionally, values that reach the lower magnitudes among the seronegatives may indicate waning antibody titers as a result of decreased exposure over long periods of time. However, there are a very small number of titer differences that reach large negative magnitudes. As this is over a six month period, drops in lnMFI-bg at these magnitudes may be due to machine reading errors, whether an individual's immune system was already declining in malarial antibody titers, receiving treatment, variable half-lives among malarial IgG (96), or other unknown physiological factors that accelerate clearance. Future research can assess these outliers further to understand the error included in MFI-bg differences using Luminex assays.

Other novel results from this study are the immune response profiles, which describe trends among individuals more than among a population. These antibody profiles for the Senegal cohort do not assume that malaria transmission only occurs during high-transmission seasons between paired visits, which is a limitation for the other previously described methods. For this analysis we selected five individuals that displayed diverse immune response profiles over the study to describe a variety of possible trends (Figure 7). Participant 1 started with high MFI-bg levels at visit 1 which decreased by visit 2. From visit 2 to visit 3 they were likely exposed to malaria, resulting in an increase in anti-PfMSP1 IgG titers that persisted at high levels from visits 3 to 7. Their MFI-bg levels may have persisted due to continued exposure to malaria between visits that sustained antibody responses, or due to persistence of IgG over time. Participant 2 sustained high MFI-bg values from visit 1 to visit 6, also possibly due to sustained exposure or persisting titers, and likely dropped between Visit 6 and 7 due to a loss in antibodies over time. If we consider Participant 2 as consistently exposed from visits 1 to 6 then the loss in antibodies over one transmission season may be explained by short malarial IgG half-lives. Alternatively, if we consider the sustained MFI-bg from visits 1 to 6 the result of long malarial IgG half-lives, then the drop between visits 6 and 7 may represent natural waning of long-lasting IgG in the absence of exposure over time. Discerning which of these two scenarios are more likely is difficult, as evidence exists for both short and long-lasting malarial IgG responses in areas of low transmission (93, 97). Participant 3 appeared to avoid exposure between visit 1 and visit 5, then experienced exposure(s) between visit 5 and 6. Participant 4 likely experienced an initial exposure between visit 1 and visit 2, and avoided exposure for the remaining

visits evidenced by continual decrease in MFI-bg until sustaining at low levels. Participant 5 may represent someone who was not exposed to malaria for the entire cohort as they did not see changes in MFI-bg, and remained below the seropositivity threshold the entire time.

Results such as these can provide personalized data on malaria epidemiology, such as the timing of previous exposure, detection of subclinical infections that elicit immune responses, or the amount of time required for antibody titers to wane once an individual is no longer exposed. It is important to fully comprehend antibody dynamics in areas of low transmission in order to answer these hypotheses. This may be accomplished by enrolling individuals who are confirmed positive for malaria at the start of this follow-up period, and then mapping their immune responses as described. A limitation to this method is that each antibody profile will vary based on the individual. For example, the rate at which IgG titer increases or decreases may depend on the person's immune response or rate at which antibodies are cleared, malarial drug treatments, or existing malaria control interventions (reviewed in (93)). Further analysis and standardization of these immune response profiles may provide a useful tool for estimating MTI in the future.

Historically, diagnostic tools have been used for malaria surveillance and to describe MTI in an area. Observing immune responses to malaria in a population with serologic assays offers an alternative surveillance method that captures previous exposures. We described the dynamics of immune responses to six malarial antigens in an endemic population using Luminex® multiplex assays. Our analyses on these

responses have shown potential estimates for MTI of four human malaria species in Thies, Senegal. It has accomplished this despite declining malaria transmission in the region through vector control efforts, which has been a major barrier for diagnostic-based surveillance. However, these techniques are untested in other populations and require further studies and standardization before expanded use in malaria surveillance. As a result, this study has provided a foundation for future serology studies, which may apply these novel estimates of MTI to other malaria-endemic regions. It also introduced limitations future studies may address, such as the influence of seropositivity cutoffs on transmission estimates and the need to understand antibody dynamics to accurately estimate malaria exposure using serology. These future studies may explore dynamics of malaria IgG further by observing longitudinal trends in immune responses for individuals who are confirmed positive for malaria in order to measure how long each IgG response persists after infection. Once these limitations are addressed, the use of serosurveillance for malaria will allow public health professionals to understand and target areas of sustained transmission regardless of transmission levels, and contribute to successful elimination efforts. At present, serologic assays such as Luminex have the potential to enhance existing malaria surveillance systems, especially as regions reduce MTI.

Public Health Implications

- This study provides a foundation for serology as a surveillance tool to measure malaria transmission intensity in a novel way.
- The results from this study demonstrate the use of serology as a specific surveillance tool different from diagnostic methods, which is capable of estimating malaria exposure in areas of low transmission intensity.
- Results from the diagnostics used at cohort visits indicate sporadic, low levels of malaria circulating in Thies, yet the serology results indicate that there is likely sustained transmission in the region.
- This study has also demonstrated the presence of multiple species of malaria in Senegal, which require different case management and surveillance strategies than those currently used in the area.
- The proposed novel analyses provide alternative analyses for serologic studies, which have historically used SCR models to describe malaria transmission intensity.
- Researchers are able to perform these assays with only a small amount of blood on filter paper, which can reduce costs for serology studies by removing the need for trained medical staff and storage.
- The use of dried blood spots means blood collected at any time for any purpose can be analyzed using the described methods, which expands the potential areas that can benefit from serosurveillance.

- Obtaining blood spots for serosurveillance is less resource-intensive compared to sample collection for diagnostic-based surveillance, and can be incorporated into other public health programs.
- The identification of four malaria species in Thies using these methods demonstrates the ability to surveil multiple species of malaria within one assay to better inform malaria control efforts in co-endemic regions.
- Success in differentiating species of malaria demonstrates the potential for serosurveillance to integrate multiple infectious diseases into surveillance programs, including viruses, bacteria, and parasites.
- Optimized serosurveillance can strengthen existing surveillance systems that rely on diagnostics, resulting in more effective response to malaria by public health professionals.
- Regions working towards malaria elimination may use the robust measures of malaria transmission intensity obtained by antibody responses as parasite levels decrease, which can help in the overall success of elimination efforts.

Tables and Figures

Table 1: Cohort Visit Sampling Timeframe

Year	2012		2013		2014		2015
Visit Number	1	2	3	4	5	6	7
Months Sampled	Aug - Sep	Jan - Feb	Aug - Sep	Jan - Feb	Aug - Sep	Jan - Feb	Aug - Sep

Table 2: Thies Cohort Demographics Across Visits

	2010		2011		2012		2013
Visit	1	2	3	4	5	6	7
n	1478	1220	899	1249	895	1146	799
Sex							
Female	737 (56%)	697 (57%)	519 (58%)	728 (58%)	512 (57%)	680 (59%)	470 (59%)
Age group at baseline							
0-4	269 (20%)	244 (20%)	181 (20%)	239 (19%)	169 (19%)	222 (19%)	65 (8.1%)
5-10	193 (15%)	181 (15%)	141 (16%)	217 (17%)	158 (18%)	204 (18%)	188 (24%)
11-15	168 (13%)	164 (13%)	114 (13%)	170 (14%)	118 (13%)	175 (15%)	122 (15%)
16-20	134 (10%)	130 (11%)	85 (9.5%)	128 (10%)	77 (8.6%)	109 (10%)	75 (9.4%)
21+	561 (42%)	501 (41%)	378 (42%)	495 (40%)	373 (42%)	436 (38%)	349 (44%)
Anemia*							
Mild	375 (28%)	287 (24%)	207 (23%)	257 (21%)	227 (25%)	250 (22%)	‡
Moderate	294 (22%)	216 (18%)	241 (27%)	100 (8.0%)	106 (12%)	174 (15%)	‡
Severe	11 (0.7%)	17 (1.4%)	44 (4.9%)	5 (0.4%)	6 (0.7%)	21 (1.8%)	‡
Self-Reported Fever (within past 24 hours)	32 (2.2%)	53 (4.3%)	45 (5.0%)	25 (2.0%)	46 (5.1%)	65 (5.7%)	‡
Identified Malaria Cases							
Microscopy†	1 (0.1%)	12 (1.0%)	1 (0.1%)	2 (0.2%)	1 (0.1%)	4 (0.4%)	0 (0.0%)
RDT	1 (0.1%)	9 (0.7%)	3 (0.3%)	17 (1.4%)	3 (0.3%)	4 (0.4%)	3 (0.4%)

*Severity of anemia defined by WHO criteria (see Appendix B)
†Cases included if two microscopists independently provided a positive diagnosis
‡Not collected at this visit

Table 3: Frequency and Proportion of Increases in MFI-bg Above Seropositive Cutoff, 2012-2014 Seasons

	2012			2013			2014		
Visit	1	2		3	4		5	6	
n	1477	1253	%Δ	985	1311	%Δ	909	1223	%Δ
Frequency of Seropositives									
P. falciparum									
MSP-1	381 (26%)	510 (41%)	14.0%	399 (41%)	346 (26%)	-15.0%	303 (33%)	449 (29%)	-4.0%
AMA-1	375 (25%)	275 (22%)	-3.0%	230 (23%)	292 (22%)	-1.0%	167 (18%)	226 (19%)	1.0%
LSA-1	28 (1.9%)	31 (2.5%)	0.6%	24 (2.4%)	34 (2.6%)	0.2%	17 (1.9%)	21 (1.7%)	-0.2%
P. vivax									
MSP-1	2 (0.1%)	0 (0.0%)	-0.1%	2 (0.2%)	3 (0.2%)	0.0%	2 (0.2%)	7 (0.6%)	0.4%
P. ovale									
MSP-1	4 (0.3%)	14 (1.1%)	0.8%	4 (0.4%)	6 (0.5%)	0.1%	5 (0.6%)	3 (0.2%)	-0.4%
P. malariae									
MSP-1	17 (1.2%)	10 (0.8%)	-0.4%	14 (1.4%)	15 (1.1%)	-0.3%	17 (1.9%)	23 (1.9%)	0.0%

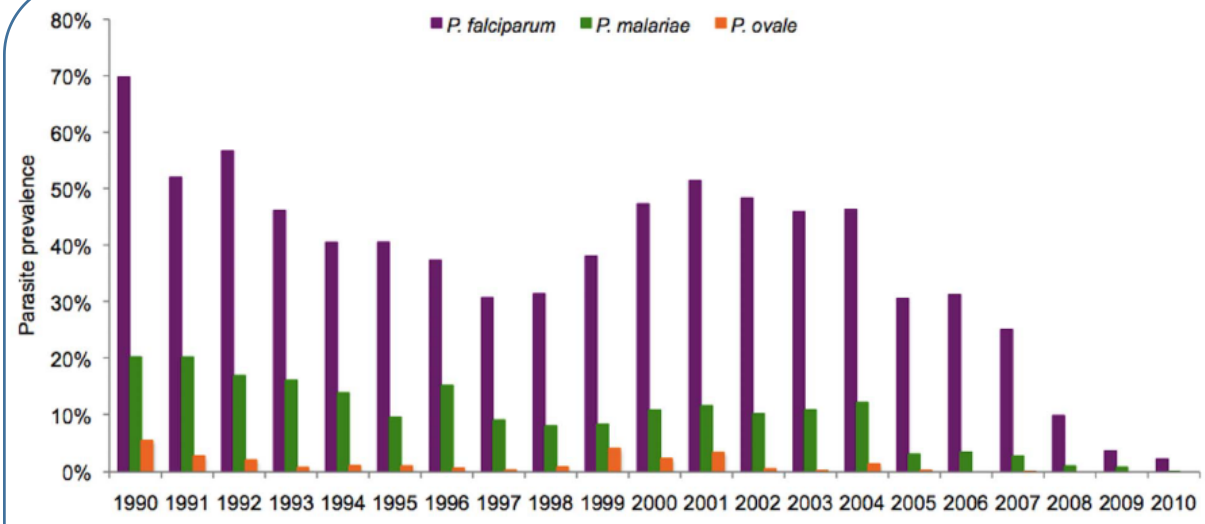

Figure 1: Parasite Prevalence Measured by Microscopy in Dielmo, Senegal Displays Variable Trends in Transmission of Three Malarial Species from 1990-2010 (adapted from (29)). Cases of malaria in Dielmo were detected year-round using both active and passive disease surveillance for village population. Sample size range = 247 (1990) to 468 (2010).

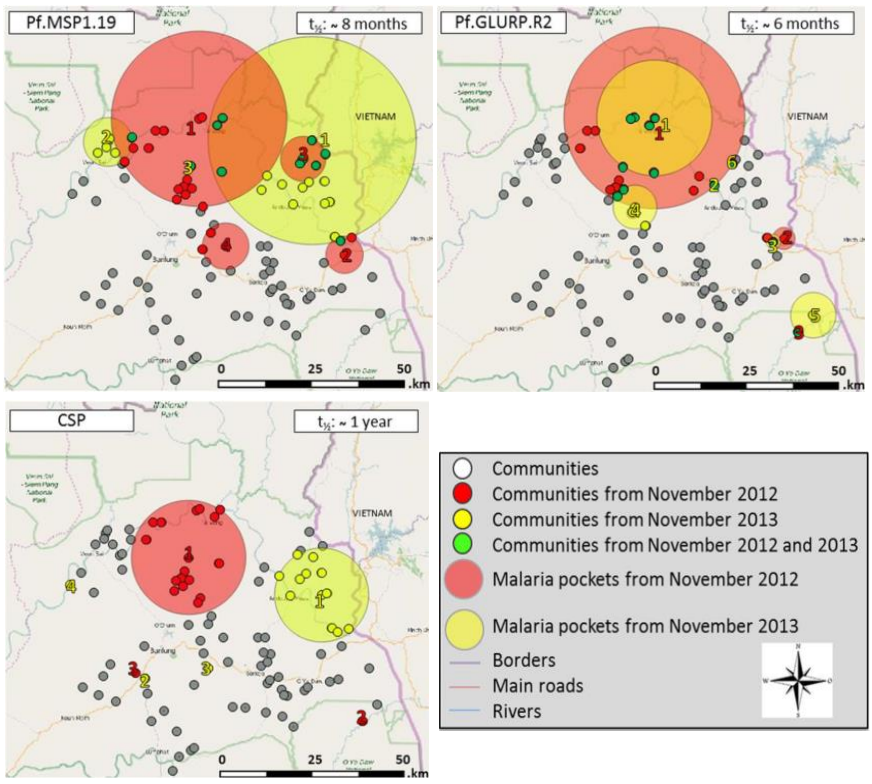

Figure 2: Various Distributions of *P. falciparum* Hotspots Identified by Several Antibody Responses Using Luminex Assays (Adapted from (84)). Maps display clustering of malaria cases for years 2012 (n = 3264) and 2013 (n = 3238) in a holoendemic region of Cambodia.

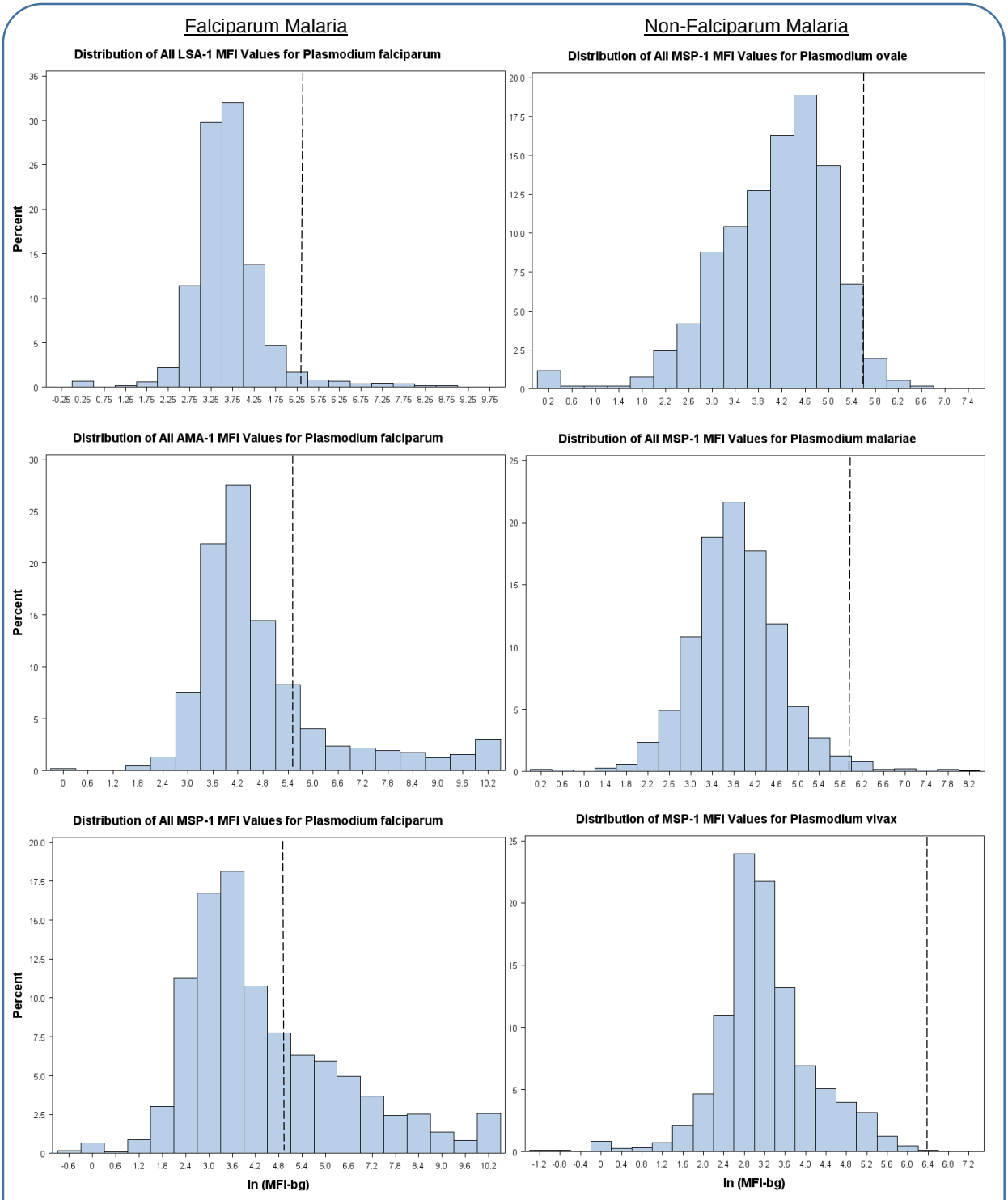

Figure 3: Distributions of lnMFI-bg for Plasmodia species by IgG in Thies, Senegal 2012-2015. Hashed lines in the histograms represent cutoffs at which values that fall above are considered 'seropositive' for malaria. The left column displays values for *P. falciparum*, and the right column displays values for *P. ovale*, *P. malariae*, and *P. vivax*. All values for MSP-1 are for the specific MSP-1_{19} epitope.

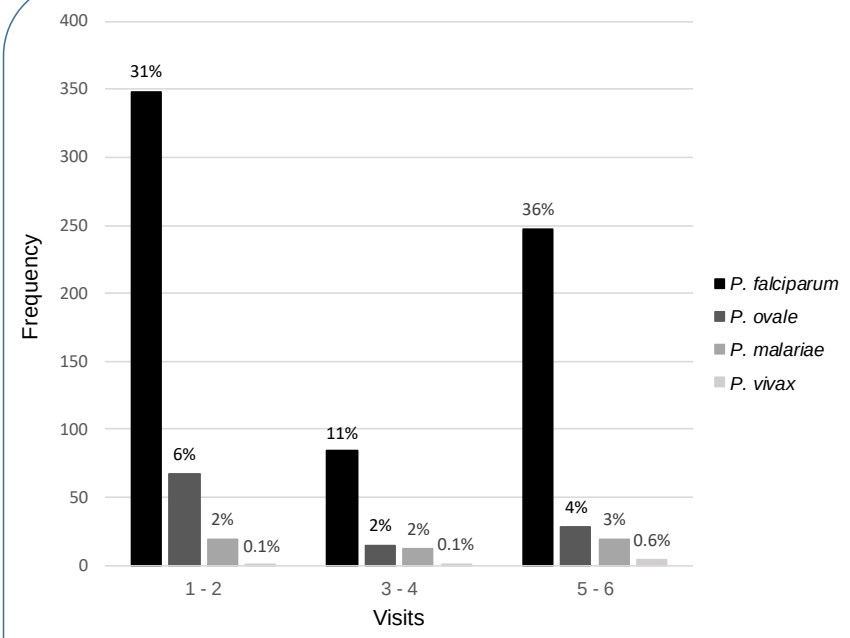

Figure 4: Frequency and Percent Increase of Positive Changes in MSP-1_{19} MFI-bg Among Individuals by Malaria Species. Bars represent the number of individuals whose MFI-bg levels have increased across paired visits by any amount and are above the seropositivity cutoffs. Percentages above bars represent the proportion of each sample that experienced an increase in MFI-bg.

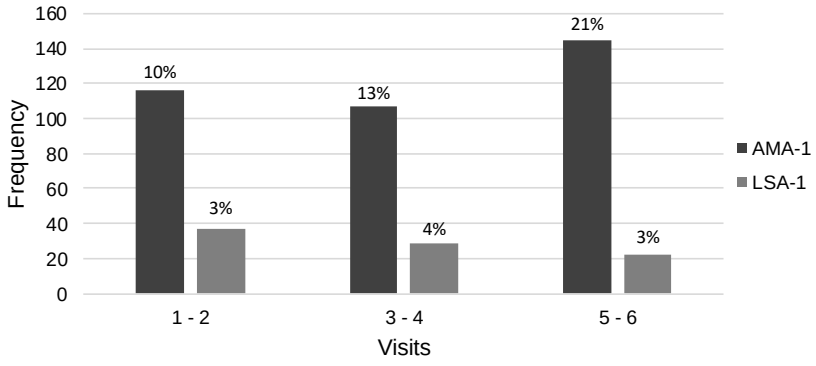

Figure 5: Frequency and Percent Increase of Positive Changes in AMA-1 and LSA-1 MFI-bg Among Individuals for *P. falciparum*. Bars represent the number of individuals whose MFI-bg levels have increased across paired visits by any amount and are above seropositivity cutoffs. Percentages above bars represent the proportion of each sample that experienced an increase in MFI-bg. Note both IgG responses are for *P. falciparum*.

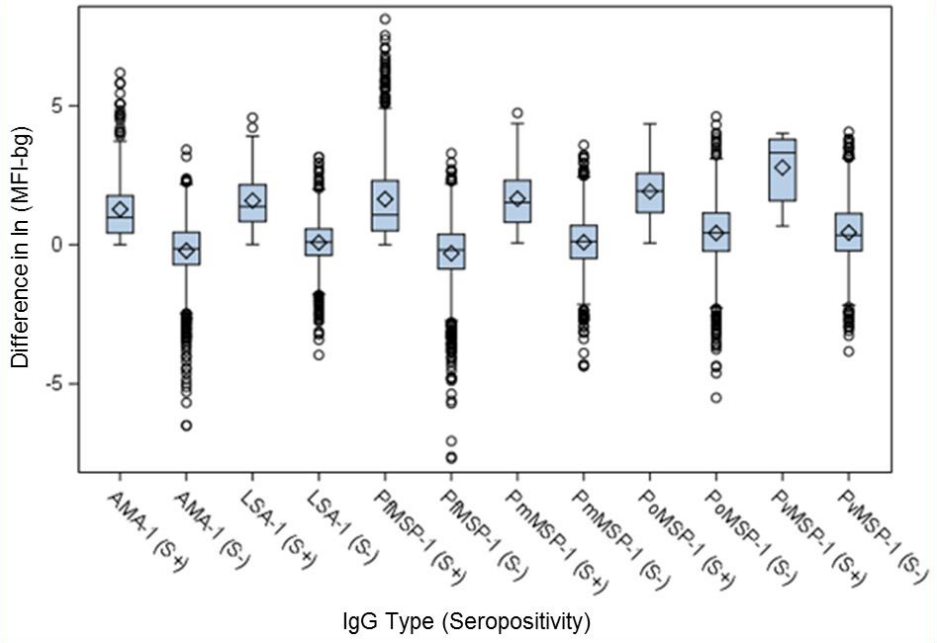

Figure 6: Distribution of Changes in ln (MFI-bg) From All Paired Visits by IgG Type and Seropositive Status. Plotted differences were calculated by subtracting lnMFI-bg values of the pre-transmission season visits from the post-transmission season visits (n = 15,565). Seropositivity determined if the post-transmission season lnMFI-bg fell above determined seropositivity cutoffs.

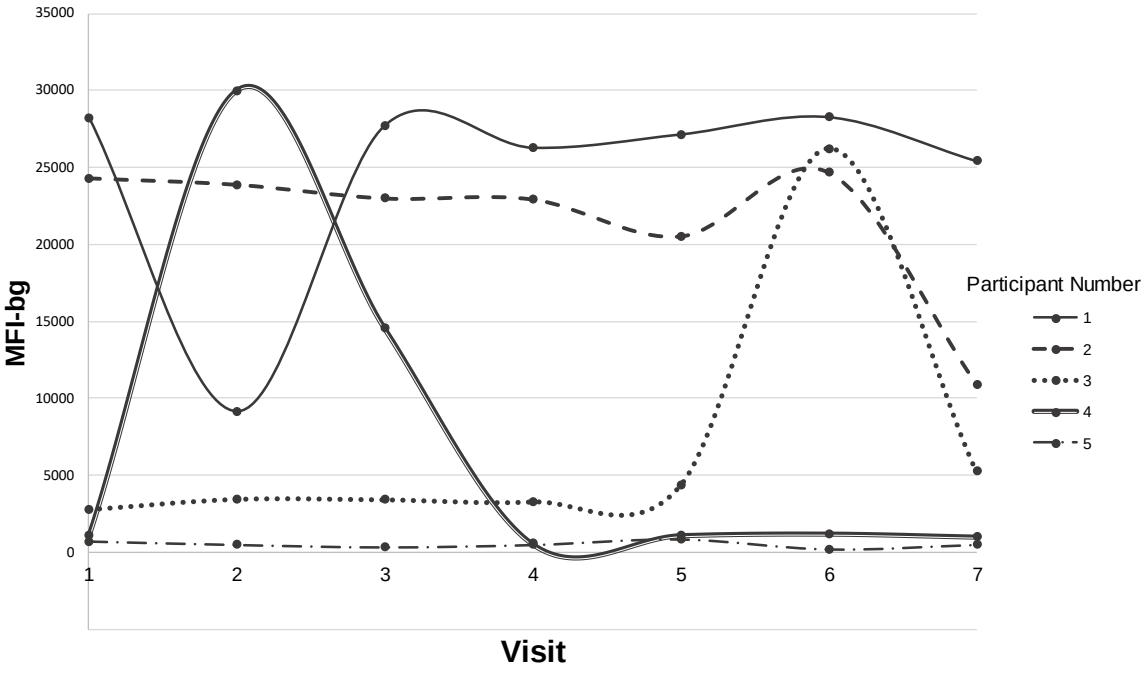

Figure 7: Dynamics of IgG Titers Against PfMSP-1_{19} Estimated by MFI-bg for Five Individuals Across All Cohort Visits in Thies, Senegal 2012-2015. Individuals are distinguished by differing line styles.

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Appendices

Appendix A: Data Organization Figures

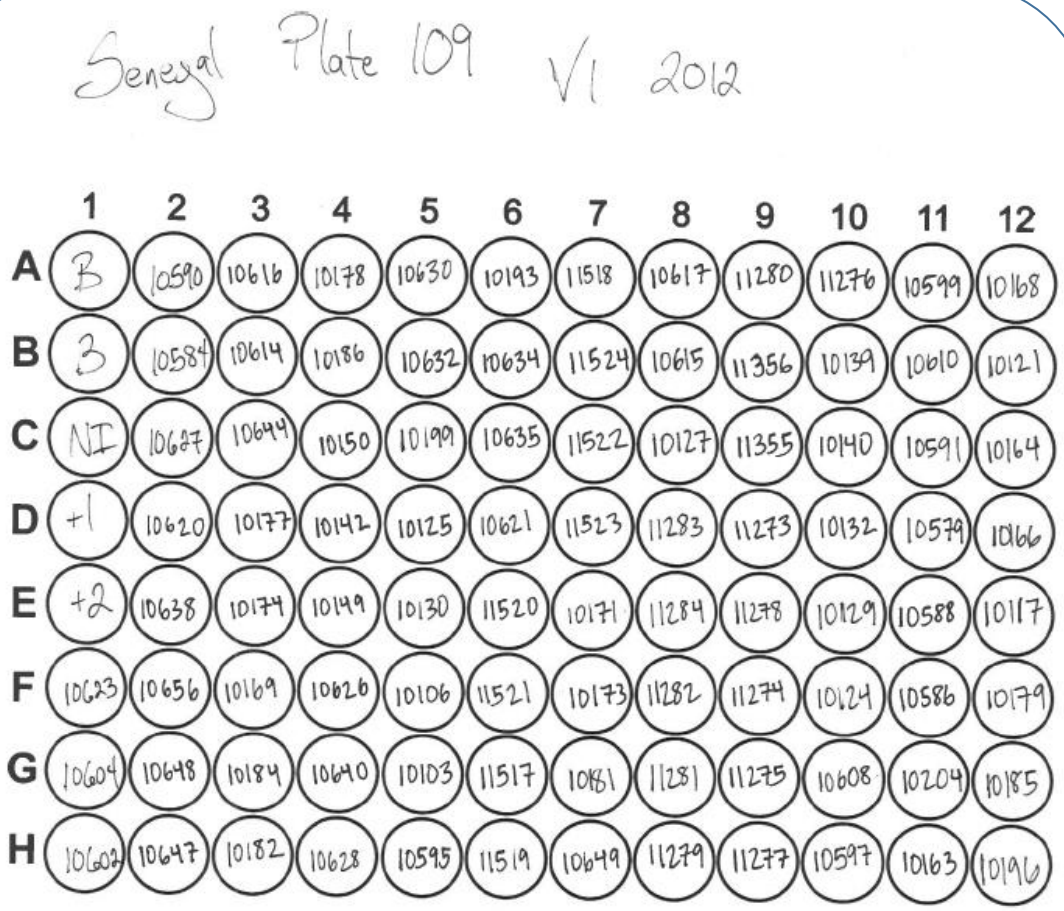

Figure A1: Sample plate setup for Luminex assay

	A	B	C	D	E
1	Input_Order	Plate_location	ID_number	followup_survey_number	Notes
2	1	F1			
3	2	G1			
4	3	H1			
5	4	A2			
6	5	B2			
7	6	C2			
8	7	D2			
9	8	E2			
10	9	F2			
11	10	G2			
12	11	H2			
13	12	A3			
14	13	B3			
15	14	C3			
16	15	D3			
17	16	E3			
18	17	F3			
19	18	G3			
20	19	H3			
21	20	A4			
22	21	B4			
23	22	C4			
24	23	D4			
25	24	E4			
26	25	F4			
27	26	G4			
28	27	H4			
29	28	A5			
30	29	B5			
31	30	C5			
32	31	D5			
33	32	E5			

Figure A2: Template for organizing serology plate data

Appendix B: Cohort Study Protocol

The following protocol for field procedures and laboratory analysis is provided with permission from CDC Malaria Branch.

Funding and Ethics

Supplies and personnel support for the bi-annual surveys in Theis, Senegal were funded by a West Africa ICEMR award from the National Institute of Allergy and Infectious Diseases (NIAID, Bethesda, MD)(DMID Funding Mechanism: U19 AI 089696-03). Supplies for laboratory data collection at CDC-Atlanta were provided by the CDC's Malaria Branch.

The survey protocol was approved by the University Cheikh Anta Diop (Dakar, Senegal, FWA 00003099) IRB for field implementation, and by the CDC IRB for non-engagement status in human subjects research.

Study Site and Participants

The population of Thies, Senegal was selected for prospective cohort bi-annual sampling in order to investigate the seasonal changes to the epidemiology of malaria in this area, and to make predictions about malaria transmission in Senegal as a whole. Up to 2,000 participants were selected from the site by household recruitment, and volunteers of all ages were selected randomly to participate in the cross-sectional surveys and subsequent cohort studies because they resided in a community from which cases of uncomplicated *P. falciparum* malaria have been reported in the previous years based on health center records. Inclusion criteria included: 1) Random selection of the subject's home within a village/community identified as having cases of

uncomplicated *P. falciparum* malaria transmission during previous years (based on health center records for 2010), 2) Potential subject expects to remain primarily in the study area during the next 18 months; and persons were excluded if 1) Pregnancy nor breast feeding in women, 2) Chronic heart or kidney disease, cancer, diabetes or other life-limiting or –threatening chronic diseases.

Upon written informed consent (or assent if under 15 years of age), persons were requested to fill out a questionnaire asking for general personal data, treatment-seeking behavior, and if their household owned (and used) a bednet. Brief histories and physical examinations were performed on each individual participant by local investigators approved to perform those procedures in Senegal. Both the histories and physicals were focused on malaria for history for fever, chills, headache, myalgias, antimalarial and other medications; physical examination for vital signs [blood pressure, heart rate, temperature], with brief cardiac and lung examinations, plus abdominal palpation for splenomegaly. Participants or their caregivers (for children) were advised to go to the community/health center/village health worker (CHW) identified as being closest to their home any time they or their child became sick. All participants had a malaria rapid diagnostic test (RDT) performed to check for malaria infection. In addition, a thick smear and a blood sample on filter paper (FTA cards, GE Healthcare) for genotyping and serology were prepared. If the RDT was positive, a full clinical examination was performed, and the subject was treated with artemisinin-combined therapy (ACT) according to national guidelines.

As part of the prospective cohort design, households were visited twice a year in attempt to locate the same individuals for questionnaires, examinations, malaria

diagnostic tests, and filter paper collection. Sampling timeframe was strategically selected to coincide before and after the rainy seasons in Thies: pre-season, August-September; post-season, January-February. Previous internal data from Senegalese Ministry of Health had shown strong correlation of rainy season with expansion of the *Anolpheles* vector population and malaria transmission. Upon initial enrollment in 2011 or 2012, all participants were attempted to be reached at each subsequent visit, regardless if they had missed previous samplings.

Blood elution, dilution, and serology data collection with Luminex® system

A 6mm circular punch was taken from the center of each filter paper blood spot, corresponding to 10uL whole blood, for elution. Samples incubated overnight in 200uL elution buffer containing: PBS (pH 7.2), 0.05% Tween-20, 0.05% sodium azide, and stored at 4^{o}C until analysis. Elution from blood spots provided an initial 1:20 dilution, and samples were further diluted 1:10 in Luminex sample diluent for a final whole blood dilution of 1:200, corresponding to a serum dilution of approximately 1:400 with the assumption of 50% hematocrit in whole blood. For Luminex sample diluent, samples were diluted in a buffer containing 0.5% Polyvinyl alcohol (Sigma), 0.8% Polyvinylpyrrolidine (Sigma), 0.1% casein (ThermoFisher), 0.5% BSA (Millipore), 0.3% Tween-20, 0.1% sodium azide, and 0.01% *E. coli* extract to prevent non-specific binding.

Three *P. falciparum* antigens were employed: the 19kD fragment of merzoite protein 1 (MSP-1_{19}) fused to glutathione S-transferase (GST) cloned from *P. falciparum* isolate 3D7, the external domain of Apical Membrane Antigen- 1 (AMA-1), and the

Pl1043 epitope from *P. falciparum* liver stage antigen 1 (LSA-1). Antigens were coupled to BioPlex® COOH beads (BioRad, Hercules, CA) according to manufacturer's protocol in the presence of 50 mM 2-(4-morpholino)-ethane sulfonic acid, 0.85% NaCl at pH 5.0 and an antigen concentration of 30 ug/mL for MSP-1_{19}, 20ug/mL for AMA-1 and 60ug/mL for LSA-1. Sulfo-NHS was purchased from ThermoFisher and EDC from Sigma-Aldrich. As a control to test for any serum IgG against GST, a bead was included in the panel which was coupled to GST at a concentration of 20ug/mL.

Reagent diluent (Buffer C) consisted of PBS-T plus 0.5% BSA, 0.02% sodium azide. Filter bottom plates (Multiscreen 1.2 µm, Millipore) were pre-wetted with PBS-T and 1,500 beads/analyte incubated with sample in duplicate for 1.5h under gentle shaking. Secondary antibodies tagged with biotin (1:500 anti-human IgG_{1-3}, Southern Biotech, Birmingham, AL; 1:2,500 anti-human IgG_4, Sigma) were incubated for 45min, and subsequent incubation with streptavidin-phycoerythrin (1:200, Invitrogen) for 30min. Plates had a final wash incubation with reagent diluent for 30min and were read on a Bio-Plex 200 machine by generating the median fluorescence signal for 50 beads/analyte. Final MFI was reported for a sample after subtracting MFI values from blank background beads that were included on each plate.

Data Analysis

Samples from persons never exposed to malaria were gathered from blood donated to a community blood bank in Memphis, TN. All blood units were from persons that had screened negative for HIV and hepatitis B viruses and had no reported history of international travel in the last 6 months. To determine seropositivity cutoff values above which the researcher had high confidence in a positive IgG signal, 92 of the US

donors were screened by all antigens used in the study, MFI-bg values were log transformed to estimate a mean and standard deviation for a ‘nonimmune’ population, and the MFI-bg cutoff value was determined by adding three standard deviations to the mean and exponentiating back to a linear scale.

Individuals were dichotomized as positive/negative for IgG against particular antigens if their MFI-bg Luminex signal was above or below the defined cutoff value for that antigen, respectively.

Table B1: Classification of Anemia by Hemoglobin Levels (g/l) (Adapted from (90))

	Anemia Classification		
Population	Mild	Moderate	Severe
Children (≤ 14 years)			
6-59 months	100-109	70-99	<70
5-11 years	110-114	80-109	<80
12-14 years	110-119	80-109	<80
Adults (≥ 15 years)			
Non-pregnant women	110-119	80-109	<80
Pregnant women	100-109	70-99	<70
Men	110-129	80-109	<80

Appendix C: SAS Code for Data Organization

```
/*CREATING DATABASES FOR EACH VISIT*/

/*VISIT 1 FULL DATASET*/
DATA THESIS.COHORT_V1;
  SET THESIS.COHORT_ALL;
  IF followup_survey_number > 1 THEN DELETE;
RUN;
*1412 OBS;

*CHECK;
PROC PRINT DATA = THESIS.COHORT_V1;
RUN;

/*VISIT 2 FULL DATASET*/
DATA THESIS.COHORT_V2;
  SET THESIS.COHORT_ALL;
  IF followup_survey_number < 2 or followup_survey_number > 2 THEN DELETE;
RUN;
*1403 OBS;

/*CHECK*/
PROC PRINT DATA = THESIS.COHORT_V2;
RUN;

/*VISIT 3 FULL DATASET*/
DATA THESIS.COHORT_V3;
  SET THESIS.COHORT_ALL;
  IF followup_survey_number < 3 or followup_survey_number > 3 THEN DELETE;
RUN;
*1394 OBS;

/*CHECK*/
PROC PRINT DATA = THESIS.COHORT_V3;
RUN;

/*VISIT 4 FULL DATASET*/
DATA THESIS.COHORT_V4;
  SET THESIS.COHORT_ALL;
  IF followup_survey_number < 4 or followup_survey_number > 4 THEN DELETE;
RUN;
*1376 OBS;

/*CHECK*/
PROC PRINT DATA = THESIS.COHORT_V4;
RUN;

/*VISIT 5 FULL DATASET*/
DATA THESIS.COHORT_V5;
  SET THESIS.COHORT_ALL;
  IF followup_survey_number < 5 or followup_survey_number > 5 THEN DELETE;
RUN;
*942 OBS;
```

```
/*CHECK*/
PROC PRINT DATA = THESIS.COHORT_V5;
RUN;

/*THERE IS NO VISIT 6*/

/*VISIT 7 FULL DATASET*/
DATA THESIS.COHORT_V7;
  SET THESIS.COHORT_ALL;
  IF followup_survey_number < 7 or followup_survey_number > 7 THEN DELETE;
RUN;
*1253 OBS;

/*CHECK*/
PROC PRINT DATA = THESIS.COHORT_V7;
RUN;

/*VISIT 8 FULL DATASET*/
DATA THESIS.COHORT_V8;
  SET THESIS.COHORT_ALL;
  IF followup_survey_number < 8 THEN DELETE;
RUN;
*1289 OBS;

/*CHECK*/
PROC PRINT DATA = THESIS.COHORT_V8;
RUN;


*Dataset created for each visit. Next step, merge using plates

-------------------------------------------------------------------------------

/*PROCEDURE FOR MERGING PLATES TO SURVEY DATABASES*/

*STEP 1: Import data from excel data (Visit: plate);

*Check that all data from Visit: plate entered successfully into SAS;

PROC PRINT DATA = WORK.PLATE149; *CHANGE TO CURRENT PLATE;
RUN;
/*-----------------------------------------------------------------
STEP 2: Make imported datasets permanent;*/

LIBNAME THESIS 'H:\Thesis\Thies_Data\SAS coding';

*Visit: plate dataset made permanent;
DATA THESIS.V8PLATE149; *CHANGE TO CURRENT VISIT: PLATE;
  SET WORK.PLATE149;
RUN;
*
-----------------------------------------------------------------
STEP 3: BEGIN MERGING (change according to the current visit/plate

MERGING V8 PLATE 149 WITH ALL COHORT INFO FOR VISIT 8;
```

```
*Ensure that the data is properly sorted by ID_number because original dataset is in that order;
PROC SORT DATA = THESIS.V8PLATE149; *CHANGE TO CURRENT VISIT: PLATE;
  BY ID_NUMBER;
RUN;

DATA VMERGE;
  MERGE THESIS.COHORT_V8 THESIS.V8PLATE149;
  BY ID_NUMBER;
RUN;

*SORT WITH INPUT_ORDER VARIABLE AFTER MERGING;
PROC SORT DATA = VMERGE;
  BY INPUT_ORDER;
RUN;

*CHECK TO SEE HOW THE SORTING WENT;
PROC PRINT DATA = VMERGE;
RUN;

* If it looks good, cut out all data that isn't pertinent to this plate;
DATA THESIS.V8PLATE149_FINAL; *CHANGE TO CURRENT VISIT: PLATE;
  SET VMERGE;
  IF INPUT_ORDER = . THEN DELETE;
RUN;

PROC PRINT DATA = THESIS.V8PLATE149_FINAL; *CHANGE TO CURRENT VISIT: PLATE;
RUN;

*STEP 4: Exported to excel
```